

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

ROLE OF RTCB PROTEIN UNDER STRESS CONDITIONS

KAAN CAN ÖMEROĞLU
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

RTCB PROTEİNİNİN STRES KOŞULLARI ALTINDA
ROLÜ

KAAN CAN ÖMEROĞLU
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

TEZ DANIŞMANI
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GEBZE

2017



YÜKSEK LİSANS JÜRİ ONAY FORMU

GTÜ Fen Bilimleri Enstitüsü Yönetim Kurulu'nun 29.06.2017 tarih ve 2017/35 sayılı kararıyla oluşturulan jüri tarafından 11.08.2017 tarihinde tez savunma sınavı yapılan Kaan...Can...Ömeroglu'ın tez çalışması Moleküler Biyoloji ve Genetik Anabilim Dalında YÜKSEK LİSANS tezi olarak kabul edilmiştir.

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...../...../..... tarih ve/..... sayılı kararı.

İMZA/MÜHÜR

SUMMARY

RTCB (RNA 2',3'-cyclic phosphate and 5'-OH ligase) enzymes are atypical RNA ligase family that joins 2'-3' cyclic phosphate or 3' phosphate termini to 5' hydroxyl termini and have different roles in cellular processes such as splicing tRNA and mRNA exons. RTCB also functions in unconventional splicing of Xbp1 mRNA, downstream element of Unfolded Protein Response (UPR).

Circadian clock is an autonomous biochemical oscillator system that facilitates to adapt environmental factors. Mammalian circadian clock system is consisted of two main parts: master clock and peripheral clock system. At molecular level, circadian clock system depends on the function of 4 genes: Clock, Bmal1, Cryptochrome 1 and Cryptochrome 2. These genes generate a transcription-translation feedback loop (TTFL). Circadian clock directly and indirectly influence almost all the cellular processes including extrinsic or intrinsic apoptosis.

Because of circadian clock regulates the main responses to cellular stresses and RTCB plays role in ER stress response and responsible for RNA ligation, we hypothesized that RTCB ligase level may be influenced by cellular stress induced apoptosis such as ER stress, oxidative stress and DNA damage. We used oxaliplatin and cisplatin for inducing DNA damage, tunicamycin for inducing ER stress and tert-Butyl hydroperoxide (tBHP) for oxidative stress. We used immunoblotting to detect the RTCB level and c-PARP level as apoptosis marker. In order to see the correlation between circadian clock system and RTCB, we used triple knockout and single clock knockout cells.

Finally, we used RTCB knockout cells to determine the stress response changes in absence of RTCB. Results indicate that neither stress induced apoptosis nor circadian clock system directly correlates with the RTCB ligase in protein level. Yet, further studies are required to understand the cellular functions of RTCB ligase.

Keywords: RTCB ligase, biological clock, ER stress, oxidative stress.

ÖZET

RTCB (RNA 2',3'-siklik fosfataz ve 5'-OH ligaz) enzimleri 2'-3' siklik fosfat veya 3'fosfat ucunu 5'hidroksil ucuna ekleyen atipik RNA Ligaz ailesidir ve trna ve mrna ekzonlarının kırılması gibi hücrel süreçlerde rol alır. RTCB ayrıca, katlanmamış protein cevabı sürecinin alt basamağında yer alan, Xbp1 Mrna'sının kırılmasında görev alır.

Sirkadiyen saat, kendi kendini kontrol eden, çevresel faktörlere uyum sağlamayı kolaylaştıran biyokimyasal bir döngüdür. Memelilerde sirkadiyen ritim iki temel parçadan oluşur: merkezi saat ve çevresel saat. Moleküler düzeyde sirkadiyen saat sistemi 4 sirkadiyen geninin işlevine bağlıdır: Block, BMal1, Cryptochrome 1 ve Cryptochrome 2. Bu genler transkripsiyon-translasyon döngüsü oluşturur. Sirkadiyen ritim iç ve dış kaynaklı apoptoz da dahil olmak üzere hemen her hücrel süreci doğrudan veya dolaylı olarak etkiler.

Sirkadiyen ritim hücrel stres cevaplarını kontrol ettiği için ve RTCB'nin er stres cevabındaki rolü ve RNA yapıştırmasındaki rolü sebebiyle RTCB ligaz proteininin seviyesinin er stres, oksidatif stres ve DNA hasarı gibi hücrel stres sonucu oluşan apoptoz sürecinde değişebileceğini öngördük. Bu sorunu aydınlatmak için oksaliplatin ve cisplatin DNA hasarı, tunikamisin ER stres ve t tert-Bütil hidroperoksit oksidatif stres oluşturmak için kullanıldı. RTCB seviyesi immunoblot ile ölçüldü. C-PARP seviyesi apoptoz belirteci olarak kullanıldı. Sirkadiyen ritim sistemi ve rtcb arasındaki ilişkiyi göstermek için üçlü knockout ve tekli knockout hücreler kullanıldı

Son olarak, RTCB yokluğunda stres cevabı değişikliklerini ölçmek için RTCB knockout hücreleri kullandık. Elde edilen sonuçlar, stresin teşvik ettiği apoptoz ve biyolojik saat sisteminin RTCB ligaz protein seviyeleri ile ilişkisinin olmadığını göstermektedir. Bununla birlikte, RTCB ligazın hücrel işlevlerinin anlaşılması için çalışmalara ihtiyaç duyulmaktadır.

Anahtar kelimeler: RTCB ligaz, biyolojik saat, ER stresi, oksidatif stres.

ACKNOWLEDGEMENTS

First, I would like to thank my parents Kenan and Belgin Ömeroğlu for their support.

I would like to thank my thesis supervisor Assoc. Prof. Dr. Nuri Öztürk for his guidance; and my colleagues Tuba Korkmaz, Handan Emiřoğlu and Gözde Özçelik for their help. I would also like to thank my colleagues Feraye Hatice Canbaz and Hüseyin Gül.

I also would like to thank Utku Özbey and Başak Aru for their helps during the writing and data analysis processes of this study.

This study was supported by the Scientific and Technical Research Council of Turkey (TUBITAK) under Grant No. TUBİTAK-114S446 and TUBA-GEBİP 2015 (to Dr. Nuri Ozturk)

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
<u>and Acronyms</u>	
μl	Microliter
μM	Micromolar
Ca^{2+}	Calcium
H_2O	Water
H_2O_2	Hydroxyl peroxide
Mg^{+2}	Magnesium
mL	Mililiter
Mn^{+2}	Manganase
O_2^-	Superoxide
$\text{OH}\cdot$	Hydroxyl radical
nm	Nanometer
Pt	Platinum
V	Voltage
AIF	Apoptosis Inducing Factor
APAF1	Apoptotic protease-activating factor 1
ASK1	Apoptosis signal-regulating kinase 1
ATF6	Activating Transcription Factor 6
ATP	Adenosine triphosphate
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
bHLH-PAS	Basic helix-loop-helix-PER-ARNT-SIM bHLH-PAS
BMal1	Aryl hydrocarbon receptor nuclear translocator-like protein 1
bZIP	Basic Leucine Zipper Domain
CAD	Caspase-Activated DNase
CCG	Clock controlled genes
CHOP	CCAAT/Enhancer-Binding Protein Homologous Protein

c-JUN	Transcription factor AP-1
Cry	Cryptochrome
DDX1	DEAD-box helicase-1
EGF	Epidermal growth factor
eIF2 α	Eukaryotic Initiation factor 2 α
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERO1	ER oxidoreductase 1
GMP	Guanosine monophosphate
GPx	Glutathione peroxidase
GTP	Guanosine triphosphate
HCC	Hepatocellular carcinoma
His	Histidine
HRP	Horseradish peroxidase
ICAD	Inhibitor of caspase-Activated DNase
IRE1	Inositol-requiring enzyme-1
MAPK	Mitogen- activated protein kinase
MOMP	Mitochondrial outer membrane permeabilization
NER	Nucleotide excision repair
NFE2L2	Nuclear factor and erythroid 2-like 2
NF κ B	Nuclear factor kappa B
PAGE	Polyacrylamide gel electrophoresis
PDI	Protein disulfide isomerase
Per	Period
PERK	PRKR-Like Endoplasmic Reticulum Kinase
ROS	Reactive oxygen species
Rpm	Rotation per minute
RTCB	RNA 2',3'-cyclic phosphate and 5'-OH ligase
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase

tBHP	tert-Butyl hydroperoxide
Tlr1	tRNA ligase
TNF	Tumor necrosis factor
Tpt1p	tRNA phosphotransferase 1
TRAF2	TNF receptor-associated factor 2
TSEN2	tRNA Splicing Endonuclease Subunit 2
TTFL	Transcription-translation feedback loop
UPR	Unfolded protein response
UV	Ultraviolet
XBP1	X-box binding protein1

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1. INTRODUCTION

To date, interaction between RTCB, which is an atypical RNA ligase and DNA damage induced apoptosis and cellular stress induced apoptosis has not been known. In this study, we aimed to reveal possible cellular functions of RTCB ligase in case of cellular stress.

1.1. The Purpose, Contribution and Content of Thesis

In this study, we aim to find out possible functions of mammalian RTCB on DNA damage, ER stress and oxidative stress *in vitro*. For this purpose, we induced DNA damage with oxaliplatin or cisplatin, endoplasmic reticulum (ER) stress with tert-Butyl hydroperoxide (tBHP) and oxidative stress with tunicamycin. Hepatocellular carcinoma (HCC) cell lines were selected for determining effects of the drugs indicated above on RTCB protein levels. Experiments were continued with p53 knockout and triple knockout (Cry1, Cry2 and p53) immortalized mouse fibroblast cells, provided by Dr. Nuri Öztürk. Also RTCB knockout HEK293T cells were provided by Dr. Nuri Öztürk; and effect of RTCB on cellular stress and DNA break was evaluated at the protein level.

2. LITERATURE REVIEW

2.1. RTCB Ligase

RTCB (RNA 2',3'-cyclic phosphate and 5'-OH ligase) enzymes are atypical RNA ligase family that joins 2'-3' cyclic phosphate or 3' phosphate termini to 5' hydroxyl termini [Tanaka et. al. 2011; Desai et. al. 2013; Das et. al. 2013; Huang and Pommier, 2013]. Cellular functions of RTCB are recently discovered such as splicing the tRNA and mRNA exons with 2'-3' cyclic phosphate ends and 5' hydroxyl ends [Tanaka et. al. 2011; Das et. al. 2013; Huang and Pommier, 2013] and unconventional splicing of Xbp1 mRNA which is a downstream element of unfolded protein response (UPR) in endoplasmic reticulum stress [Desai et.al. 2015; Lu et.al. 2014; Kosmaczewski et.al. 2015; Jurkin et. al. 2014; Huang and Pommier, 2013]. Among these, studies reveal that RTCB ligase has additional roles and potential unclearified functions in cellular processes. For example; the inhibitory effect of RTCB in axon regeneration in neurons was explained recently [Kosmaczewski et. al. 2015]. Because of proteostasis maintenance is essential for ER maintenance and ER maintenance is essential for proper protein folding and secretion, overcoming the stress caused by unfolded proteins is critical for cellular survival and homeostasis. The role of RTCB ligase in unfolded protein response, unconventional splicing of Xbp1 mRNA is indispensable for proper response to unfolded protein stress and without the activity of RTCB ER maintenance and secretion are disrupted [Lu et.al. 2014; Jurkin et. al. 2014]. Hence, it is known that plasma cells with depleted RTCB have reduced and disorganized ER structures with severe defects in antibody secretion [Jurkin et. al. 2014]. Altogether, these findings show that RTCB ligase and tRNA ligation complex have critical roles in cellular homeostasis. It is also shown that RTCB in *Escherichia coli* can splice the 3-phosphate and 5'hydroxyl DNA break, specifically named as dirty end, which can not be spliced by DNA ligases. However, the role of RTCB in DNA repair is not shown in mammals [Das et. al. 2013; Huang and Pommier, 2013].

2.1.1. Ligation Process of RTCB

Unlike typical RNA ligases which require ATP and magnesium (Mg^{+2}), activation of RTCB depends on GTP and Mn^{+2} [Tanaka et. al. 2011; Desai et. al. 2013; Desai et. al. 2015; Das et. al. 2013]. Another unique feature of RTCB is the ability of joining 2'-3' cyclic phosphate and 5' hydroxyl ends directly unlike yeast RNA ligase enzyme Trl1 [Tanaka et. al. 2011; Desai et. al. 2013; Tanaka et. al. 2011; Jurkin et. al. 2014; Huang and Pommier, 2013]. Ligation reaction of typical ligases initiates with the charging of catalytic lysine residue adenylation in an ATP dependent step then the adenyate is transferred to the 5' phosphate end of nucleic acids and this step activates the 5'end. As the last step, 3'OH attack on the 5'phosphoanhydride, generated 3'-5' phosphodiester bond. RTCB utilizes four step pathway starting with attacking to GTP to form RTCB-(histidiny³³⁷-N)-GMP intermediate. Activated RTCB, then hydrolyzes the RNA 2'3'-cyclic-PO₄ end to 3'PO₄. Guanylate is then transferred to from His337 to the RNA 3'PO₄ to form RNA₃, pp₅, G intermediate [Desai et. al. 2013; Tanaka et. al. 2011; Desai et.al. 2015; Das et. al. 2013; Huang and Pommier, 2013; Desai et. al. 2014]. Differences between the ligation mechanisms of classical ligases and RTCB were shown in Figure 2.1 ligases [modified from Huang and Pommier, 2013].

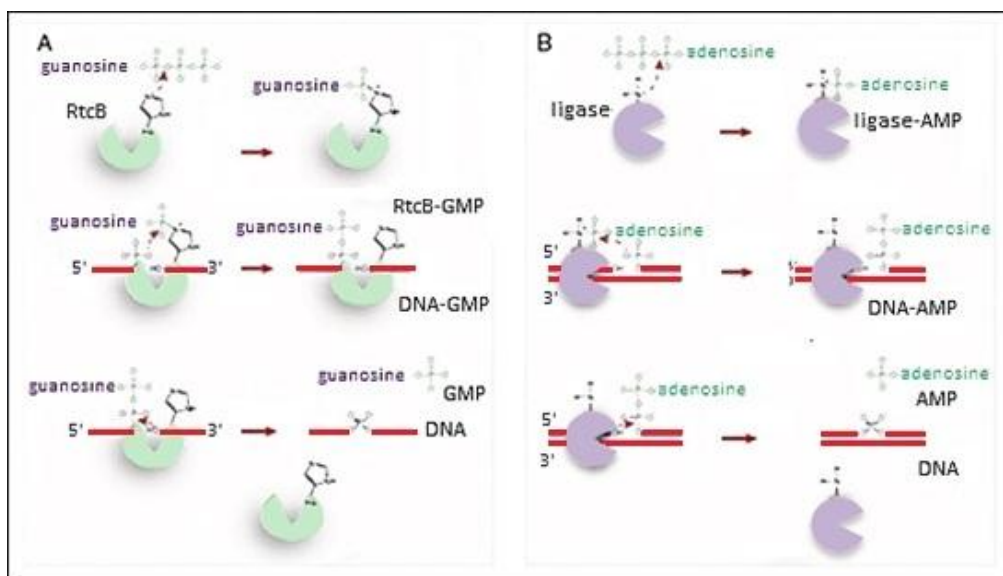


Figure 2.1: Ligation processes of a) RTCB and b) typical RNA ligases.

RTCB is the catalytic subunit of RNA splicing and ligation complex, functions as ligase. Archease, cofactor of RTCB, stimulates and accelerates the ligation process by facilitating the guanylation of RTCB which is indispensable for its function [Desai et. al. 2015; Kosmaczewski et. al. 2015; Filipowicz, 2014; Desai et. al. 2014; Popow et. al. 2014]. High conservation rate of archease and RTCB in three domains of life is occurred, in some species archease and RTCB are expressed in the same operon to constitute tRNA splicing operon [Desai et. al. 2014; Desai et. al. 2015; Popow et. al. 2014]. The surface charge of archease is anionic, it can bind to the RNA-binding site of RTCB with this anionic surface [Desai et.al. 2014; Desai et. al. 2015]. Archease also contains a metal-binding site that is essential for its action [Desai et. al. 2014]. Another function of RTCB in which archease plays role as cofactor is Xbp1 mRNA splicing in unfolded protein response. Studies indicate that Xbp1 depletion requires simultaneous knockdown of RTCB and archease shows that they function together in Xbp1 splicing and maturation [Kosmaczewski et. al. 2015; Filipowicz, 2014]. Another enzyme functions together with RTCB is TSEN2, responsible for intron cleavage and release that initiates the tRNA ligation [Kosmaczewski et.al. 2015]. Other proteins in tRNA ligase complex that play role in ligation process are still unknown, except DDX1. Interestingly, although ATP does not serve as nucleotydyl donor for RTCB, in the presence of archease, ATP stimulates guanylation of RTCB. It is shown that ATP hydrolysis by DDX1 leads to maximal activation of RTCB by archease [Popow et. al. 2014]. Among these new function of RTCB that has been recently discovered, the inhibitory function on axon regeneration is independent from its cofactors or other ligase complex members such as archease and TSEN2. In the absence of these ligase complex proteins, tRNA ligation function and Xbp1 mRNA splicing are effected negatively however absence of these proteins RTCB is still able to inhibit axon regeneration indicate that RTCB does this inhibitory function independent from tRNA ligation or Xbp1 splicing function [Kosmaczewski et. al., 2015].

2.1. Circadian Clock

Circadian clock is the molecular system that oscillates according to physiologic functions. It is autonomous and self-sustaining. In mammals, cells of peripheral organs are synchronized with each other by the signals received from the “master clock” in the suprachiasmatic nucleus (SCN), which is located in the anterior hypothalamus. Master clock’s molecular mechanism oscillates same as the peripheral clock; but SCN also can synchronise the peripheral clocks with one another and itself [Sancar et. al. 2016]. Circadian clock consists of 4 genes at the molecular level: Clock, BMal1, Cryptochrome 1 and 2, Period 1 and 2. All these genes generate a transcription-translation feedback loop (TTFL). Clock and BMal1 are bHLH-PAS domain-containing transcriptional activators; upon dimerization, they bind to promoters of Cry and Per genes to activate their transcription. Then, Cry and Per proteins make heterodimers or multimeric complexes, enter the nucleus and inhibit Clock-BMal1-activated transcription. Clock-BMal1 controls the transcription of clock controlled genes (CCGs) by causing rhythmic expression of these genes. Proteins encoded by CCGs do not directly effect the clock TTFL [Hughes, Deharo et. al. 2007]. It is also known that circadian clock is connected to both intrinsic and extrinsic apoptosis mechanisms [Sancar et. al. 2015].

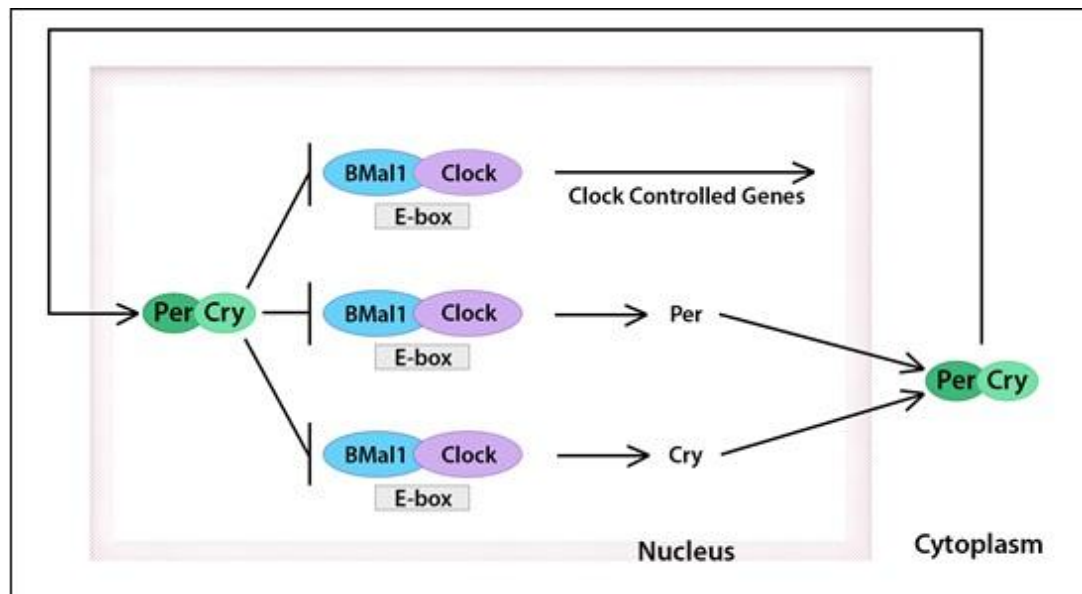


Figure 2.2: Summary of the molecular mechanism of mammalian molecular clock. Clock and BMal1 dimerizes and binds to Per and Cry genes' and other clock controlled genes' promoters, resulting in the activation of these genes. Cry and Per heterodimers enter the nucleus and inhibit the activity of Clock-BMal1 and clock controlled genes.

2.3. Apoptosis

For many years, cell death was thought as no more than the passive end of life. It was first shown in the middle of 1960s that developmental cell death relies on the activation of some endogenous genes which results in programmed cell death [Lockshin and Williams, 1964; Tata, 1966]. In 1972, studies performed by Kerr et. al. with electron microscopy for determining ultrastructural characteristics of distinct types of cell death ended up with the term "apoptosis" for defining a morphologically different type of cell death. In contrary to necrotic cells which swell and rupture due to trauma; apoptotic cells, which die during normal processes such as development and tissue homeostasis show some distinct morphological features; for example, cytoplasmic shrinkage, conservation of the integrity of membrane and organelles and nuclear condensation [Kerr et. al. 1972].

Caenorhabditis elegans, *Drosophila melanogaster* and *Mus musculus* have been studied for understanding mechanisms of programmed cell death development [Fuchs and Steller, 2015]. Apoptotic process was first studied in *C.*

elegans, in which 131 of 1090 total cells that are generated during formation of the adult worm undergo apoptosis at specific times during development, proving that apoptosis is a tightly controlled process [Elmore, 2007].

Apoptosis occurs normally as a part of development, aging; and it maintains cell population homeostasis in tissues. It is also a defense mechanism when cells are damaged due to toxic substances. There are different physiological and pathological stimulus that can end up with apoptosis; for example, chemotherapy drugs or irradiation causes apoptosis via a p53-dependent pathway. Also some hormones may cause apoptosis of particular cell types whereas not effecting, even stimulating other cell types.

2.3.1. Molecular Mechanisms of Apoptosis

Apoptosis can be triggered both intrinsically and extrinsically, and results in the activation of a special type of cysteine proteases which are synthesized as zymogens in almost all cells, caspases. Caspases are categorised as either initiator (caspase -2,-8,-9,-10) or executioner (caspase -3, -6 ve -7) caspases [Thornberry and Lazebnik, 1998; McIlwain and Berger, 2013].

Extrinsic signals initiate apoptosis with ligand-receptor interactions, which involve death receptors that belong to tumor necrosis factor (TNF) receptor gene superfamily [Elmore, 2007].

Intrinsic signals of apoptosis are non-receptor-mediated signals that trigger intracellular proteins which directly effect their targets. Intrinsic signals are mitochondria related. Intrinsic pathway can be triggered in different ways; absence of various factors may promote cell death; on the other hand, radiation, cellular toxins and free radicals also promote intrinsic apoptotic pathway. All these signals result in the loss of mitochondrial transmembrane potential, mitochondrial outer membrane permeabilization (MOMP), and release of two main group of pro-apoptotic proteins which are normally located at the intermembrane area of the mitochondria. First group of these proteins are cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi, which activate the caspase-dependent mitochondrial pathway. Cytochrome c, apoptotic protease-activating

factor 1 (APAF1), and caspase 9 pro-domain interactions forms apoptosome, the protein platform which is accepted as the initiator of apoptosis [Rodriguez and Lazebnik, 1999]. However, it has also been suggested that apoptosome is responsible for signal amplification and not for initiation of the apoptotic process. This may explain how executioner caspases can be activated despite of APAF1 and caspase 9 mutations [Srivastava et. al. 2007; Nagasaka et. al. 2010; Xu et. al. 2005]. The second group consists of AIF, endonuclease G and CAD, which are released from the mitochondria during apoptosis, however, this occurs after the cell has committed to die.

The decision point that determines apoptosis is the mitochondrial outer membrane permeabilization (MOMP), which causes cytochrome c release from the mitochondria. Control and regulation of BCL-2 family which contains both anti-apoptotic and pro-apoptotic proteins regulates MOMP: pro-apoptotic BAX (BCL-2-associated X protein) and BAK (BCL-2 antagonist/killer) oligomers comes together in the outer membrane of mitochondria; promotes membrane permeabilization and release of proteins located between membranes such as cytochrome c. This results in apoptosome formation and activation of executioner caspases; causing a cascade that ends up with the cleavage of many cellular proteins [Fuchs and Steller, 2015]. Tumor suppressor protein *p53* has a key role in regulation of the BCL-2 family [Schuler and Green, 2001].

Both intrinsic and extrinsic pathways end at the execution phase, which is begin with the activation of executive caspases. Among executive caspases, caspase-3 is considered to be the most important one. Caspase-3 activates CAD, which is normally complexed with it's inhibitor ICAD in proliferating cells. Caspase-3 cleaves ICAD and releases CAD, which results in the degradation of chromosomal DNA and chromatin condensation. PARP-1 is another cellular substrate of caspases. PARP cleavage (c-PARP) by caspases is also an indicator of apoptosis process. It is known that PARP cleavage by caspase-3 is observed in several brain diseases including brain cancers; in addition to caspase-3, caspase-7 also cleaves PARP-1 [Chaitanya et. al. 2010]. AIF translocates to the nucleus and promotes DNA fragmentation and peripheral chromatin condensation, which is named as stage I condensation. Endonuclease G cleaves nuclear chromatin. Both AIF and

endonuclease G work independent of caspases. DNA fragments of 180-200 base pairs are seen in apoptosis, which can be observed under ultraviolet exposure when stained with a nucleic acid intercalating agent after agarose gel electrophoresis. Hallmark of the final stage of apoptosis is phosphatidylserine externalization on the apoptotic cell surface. Apoptotic cells are then phagocytosed [Elmore, 2007].

2.3.2. DNA Damage Induced Apoptosis

Even healthy cells' genomic DNA integrity is under threat; since endogenous reactive oxygen species, errors during replication and recombination, or genotoxins may cause DNA damage [Norbury and Zhivotovsky, 2004]; however, it was shown that in most cases, external factors cause DNA damage. This makes DNA the main target of most anticancer agents which either directly interact with DNA, incorporate into DNA, or block DNA metabolism by inhibiting DNA polymerases or topoisomerases [Roos et. al. 2016]. DNA damaging agents do not only effect DNA but also damage other cellular components. For example, UV light and alkylating agents activate epidermal growth factor (EGF) receptor, causing a DNA-damage-independent response involving the mitogen-activated protein (MAP)-kinase pathway [Tenzer, 2002]. However, there are evidences that indicate genotoxins' apoptotic activities are mainly related with DNA damage. First, except DNA mismatch repair mutants, nearly all DNA-repair defective cells are hypersensitive to genotoxins [Christmann et al. 2003]. Second, modified nucleotide precursors can sensitize cells to undergo apoptosis. The ganciclovir model shows that a simple modified base incorporated into DNA can cause apoptosis during replication [Tomicic et al. 2002]. Finally; double strand breaks of DNA induced by the enzymes trigger apoptosis [Lips and Kaina, 2001].

One of the crosslinking agents, cisplatin mostly reacts with the N7 of guanine and forms inter- and intra-strand DNA cross-links [Jamieson and Lippard, 1999]. These lesions are repaired by nucleotide excision repair (NER), and cells that are defective in NER are more sensitive to cisplatin. Cisplatin lesions block replication and transcription, causing replication mediated double strand breaks. Also it activates c-Jun N-terminal kinase (JNK) and p38 kinase [Brozovic et. al. 2004].

Another crosslinking agent, oxaliplatin is a third generation platinum (Pt) anticancer agent. It differs from cisplatin with the diaminocyclohexane (DACH) ligand, which is an amine group in cisplatin. Oxaliplatin's antitumor activity, also without or a partial cross-resistance with cisplatin has shown on human tumors both in vivo and in vitro. Effect of all antitumor Pt drugs relies on their anti-proliferative properties. However, oxaliplatin inhibits cancer cell growth at least as potent as cisplatin. In an article, published in 2000 suggests that cytotoxic properties of oxaliplatin may be the result of proportionately more highly lethal lesions; implying oxaliplatin's ability of damaging DNA is higher than cisplatin [Wojnarowski et al. 2000].

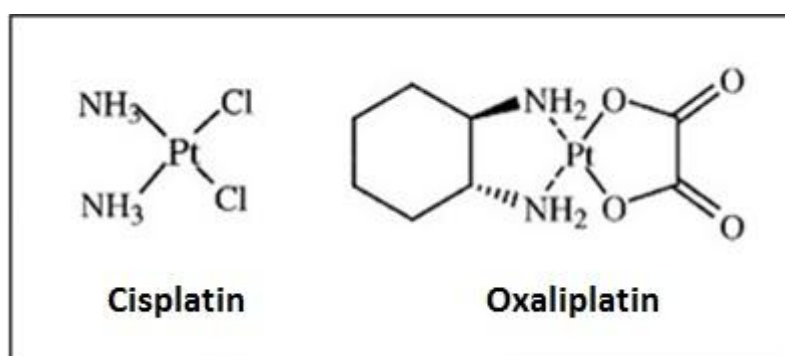


Figure 2.3: Molecular differences between cisplatin and oxaliplatin.

2.4. Endoplasmic Reticulum Stress and Unfolded Protein Response

2.4.1 Endoplasmic Reticulum Stress

Endoplasmic reticulum (ER) is an essential organelle that is responsible for translation, post-translational modification, proper folding and fate determination of many proteins including secretory proteins. ER also functions as Ca^{2+} storage. As the first step of secretory pathway, ER ensures that proteins that will be secreted are folded and modified properly by the process called quality control. While the only correctly folded proteins are exported to the Golgi apparatus, unfolded or misfolded proteins are retained in the ER to complete the folding process or degradation [Schröder and Kaufman, 2005; Breckenridge and Germain, 2003; Xiang et. al. 2017]. If the final structure of proteins remains inappropriate, unfolded proteins are translocated to the cytosol, targeted to ubiquitylation and proteasome-dependent degradation. This process is called ER-associated degradation (ERAD) [Xiang et. al. 2017]. If the misfolded proteins accumulate in the ER, this causes the protein stress or ER stress. Adaptation mechanisms against ER stress are ERAD and multiple cellular responses with the activation of unfolded protein response (UPR) elements. If the cell cannot recover the stress then one of the function of UPR elements to initiate apoptosis [Lu et.al., 2014; Filipowicz, 2014; Schröder and Kaufman, 2005; Xu et. al. 2005; Breckenridge and Germain, 2003].

2.4.2. Unfolded Protein Response (UPR)

Multiple regulatory networks have been evolved to maintain the proteostasis in ER are called UPR. Functions of UPR elements are increasing the expression and activity of ER chaperones to diminish the unfolded protein aggregate, increases the volume of ER by increasing the lipid biogenesis, focusing the chaperons to the misfolded aggregate by inhibition of transcription and translation and if these responses cannot recover the stress initiate the apoptosis [Lu et.al. 2014; Schröder and Kaufman, 2005; Xu et. al. 2005]. These functions are mediated by three

branches of UPR signal which are IRE1, PERK and ATF6. These transmembrane proteins except ATF6 are normally kept in silent state by the interaction of their N-terminal luminal domains and ER chaperone GRP78/Bip. In the case of ER stress, Bip dissociates with UPR elements to refold the misfolded aggregate. Dissociation of Bip is stress signal and initiates the downstream responses of three branches of UPR. Activation of IRE1 and PERK depend on the homooligomerization and trans-autophosphorylation. Upon activation, IRE1 excises a short sequence from X-box binding protein (XBP-1) by its C-terminal endonuclease domain generates active bZIP transcription factor to trigger the expression of ER chaperones. PERK phosphorylates the translational initiation factor eIF2 α to inactivate the translation. ATF6 is proteolytically cleaved to release its bZIP transcription factor domain [Lu et.al. 2014; Filipowicz, 2014; Schröder and Kaufman, 2005; Xu et. al. 2005; Ryoo, 2016; Breckenridge and Germain, 2003]. Signal transduction pathways upon ER stress are shown in Figure 2.4 [Kitamura M., 2008].

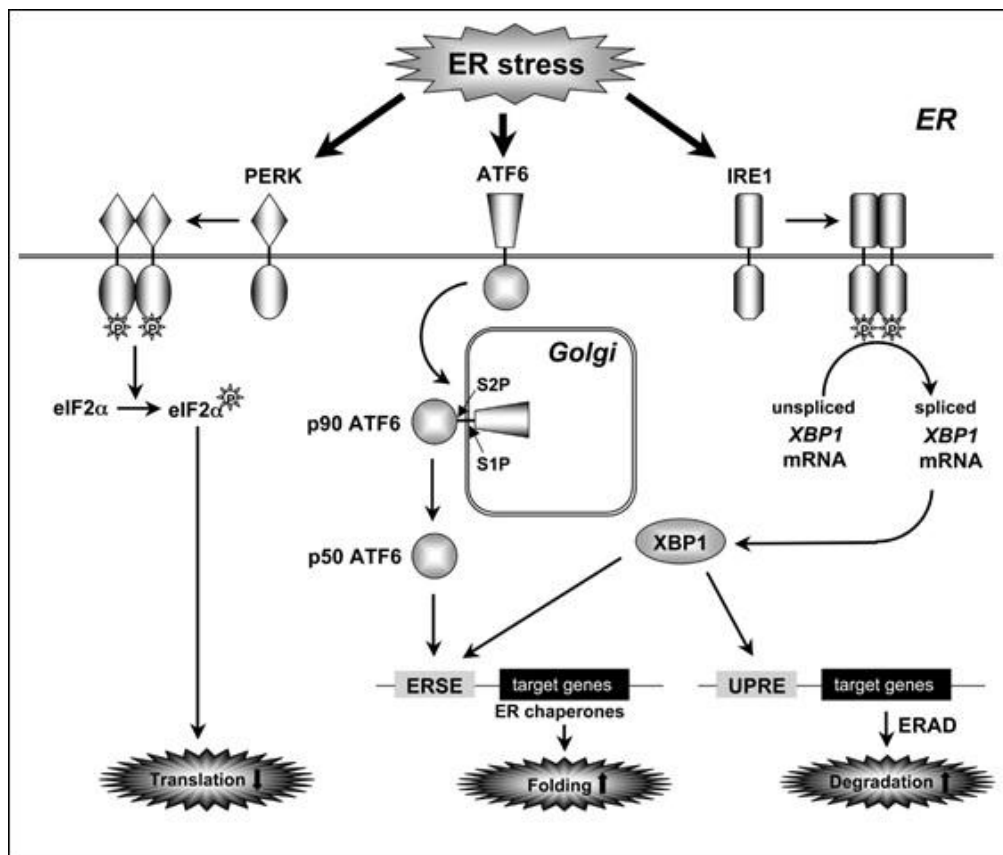


Figure 2.4: Signal transduction pathways upon ER stress.

2.4.3. Endoplasmic Reticulum Stress Induced Apoptosis

If the adaptive responses cannot recover the stress, then UPR can activate the apoptosis with both intrinsic and extrinsic pathways. BAX and BAK proteins undergo conformational change that lead to Ca^{2+} release from the ER lumen by ER-localized BAK. Release of Ca^{2+} converts pro caspase-12 to caspase-12 localized in ER and activation of caspase-12 initiates caspase cascade- continues with cleavage of procaspase-9 and -3 by caspase-9. Mitochondria takes up the Ca^{2+} released from ER results with the damage in inner membrane potential and initiate the intrinsic apoptosis. By the function of ATF6 pathway, expression of transcription factor CHOP is up-regulated. CHOP downregulates the transcription of Bcl-2 which is anti-apoptotic protein. Another apoptotic pathway induced by ER stress initiates with the formation of IRE1-TRAF2-ASK1 complex. This complex activates the c-Jun N-terminal kinase and apoptosis [Schröder and Kaufman, 2005; Xu et. al. 2005; Ryoo, 2016; Breckenridge and Germain, 2003].

2.4.4. Role of RTCB in UPR

Recent studies show that RTCB with its cofactor Archease has an essential role in UPR, ligation of Xbp1 which is cleaved with the activation of IRE1 [Lu et.al. 2014; Kosmaczewski et. al. 2014; Kosmaczewski et. al., 2015; Jurkin et. al. 2014; Filipowicz, 2014;]. IRE1 is the most conserved UPR branch and results similar breaks in mammals Xbp1 and yeast Hac1 mRNA with 2'3'PO₄ end and 5'OH end. Yeast tRNA ligase Trl1 ligates the Hac1 mRNA with multiple steps leaving a 2'phosphate in splicing site and Tpt1p then removes 2'phosphate. By contrast, RTCB ligates Xbp1 mRNA via single step reaction. Xbp1 splicing with the action of IRE1 and RTCB is indispensable for proper response to ER stress [Lu et.al. 2014; Jurkin et. al. 2014; Filipowicz, 2014; Schröder and Kaufman, 2005; Kosmaczewski et. al., 2014]. Role of RTCB in Xbp1 splicing process is shown on Figure 2.5 [Lu et al., 2014].

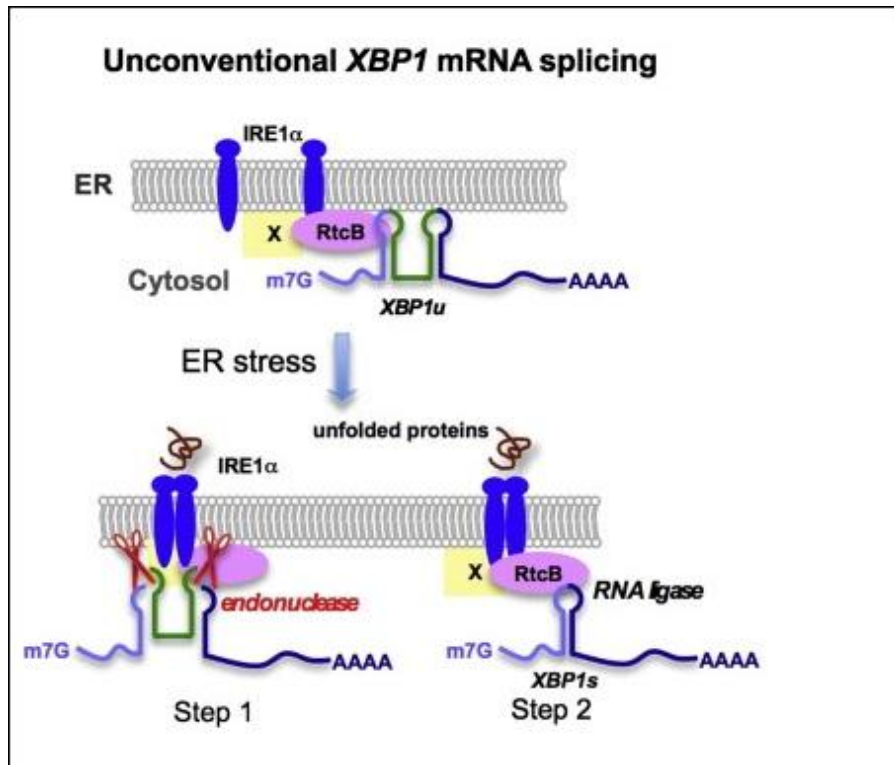


Figure 2.5: Process of Xbp1 splicing and role of RTCB in this process.

2.5. Oxidative Stress

Reactive oxygen species (ROS) and reactive nitrogen species are produced during the cellular stresses and as byproducts in cellular processes [Farooqi et. al 2015]. In normal conditions there is a balance between ROS production and ability to neutralize the reactive oxygen intermediates. Low or balanced generation of ROS facilitates the crucial cellular processes include survival, growth and maintenance of redox homeostasis [Farooqi et. al 2015; Wang et. al. 2016; Redza-Dutordoir and Averill-Bates, 2016]. Severe imbalance between ROS generation and their elimination by antioxidant mechanisms is called oxidative stress, that has been implicated with cancer, diabetes, aging and neurodegenerative diseases [Farooqi et. al 2015; Wang et. al. 2016; Liu and Ke, 2016; Redza-Dutordoir and Averill-Bates, 2016]. Mitochondria is the major intracellular source of ROS generation include superoxide (O_2^-), hydroxyl radical ($OH\cdot$), oxygen with high energy and hydroxyl peroxide (H_2O_2) [Farooqi et. al 2015; Wang et. al. 2016; Tang et. al. 2013]. In

response to ROS generation, antioxidant defense mechanism converts the superoxide into less reactive hydrogen peroxide by superoxide dismutase (SOD), then catalase, glutathione peroxidase (GPx), peroxiredoxin produce H₂O and oxygen [Wang et. al. 2016; Liu and Ke, 2016; Redza-Dutordoir and Averill-Bates, 2016]. Chronic oxidative stress causes the other cellular stress, therefore cellular responses and defense mechanisms can be compromised such as the oxidative stress induced ER stress by lipid peroxidation and disturbed ER redox state by overproduction of ROS [Farooqi et. al 2015; Wang et. al. 2016; Liu and Ke, 2016].

2.5.1. Oxidative Stress and Apoptosis

Severe accumulation of ROS leads to apoptotic action of p53 which can stabilize with the existence of ROS. Apoptosis is activated by p53 in transcriptional level with the down-regulation of anti-apoptotic proteins such as Bcl-2 and up-regulation of extrinsic and intrinsic pro-apoptotic proteins. In the case of oxidative stress, cytosolic p53 can translocate to mitochondria and interact with the pro-apoptotic proteins. This interaction leads to mitochondrial membrane permeabilization which is necessary for release of pro-apoptotic proteins to the cytosol. ROS can also directly activate the caspase cascade.

ROS is interconnected with the extrinsic apoptosis by their correlation with TNF α . ROS are able to regulate the activation of NF κ B through TNF signaling which leads to expression of antioxidant enzymes and anti-apoptotic factors. However, TNF-induced ROS accumulation inhibits the NF κ B activation and leads to apoptosis. Oxidative stress is correlated with other extrinsic apoptosis factors and pathways directly or indirectly [Redza-Dutordoir and Averill-Bates, 2016].

2.6. Interconnection Between ER Stress and Oxidative Stress

Oxidative stress and ER stress are interconnected with each other in which any of these types of stress can promote the other [Farooqi et. al 2015; Wang et. al. 2016; Liu and Ke, 2016]. Folding capacity of ER requires the oxidizing environment. Increased level of accumulated misfolded proteins and disulfide bond breakage leads to ROS accumulation and oxidative stress. In order to limit the ER stress-induced ROS accumulation one of the responses upon UPR activation is PERK-mediated phosphorylation of nuclear factor and erythroid 2-like 2(NFE2L2) to upregulate the expression of antioxidant enzymes [Farooqi et. al 2015]. Oxidative protein folding is mediated by ER protein disulfide isomerase (PDI) and ER oxidoreductase 1 (ERO1). ERO1 α domain is activated by CHOP, results with the increase in ROS production during ER stress. This type of ER stress induced-oxidative stress is a part of ER stress response that facilitates the apoptosis [Farooqi et. al 2015; Wang et. al. 2016]. Oxidative stress may induce the ER stress by disturbing ER redox state, disulfide bond formation and correct protein folding [Wang et. al. 2016; Liu and Ke, 2016]. So, accumulation of ROS can indirectly increase the production of ROS by causing ER stress and in late response, ER stress induced ROS production [Wang et. al. 2016]. Oxidative stress can also cause ER stress by releasing the Ca^{2+} from ER to cytosol that affects the redox state of protein sulfhydryl groups [Tang et. al. 2013]. This effect of oxidative stress is the same as the function mechanism of thapsigargin, a well-known ER stress inducer.

3. MATERIALS

Table 3.1: Reagents and Buffers.

10X SDS Running Buffer	250 mM Tris Base (pH:8.3) 1.9 M Glycine 1% SDS
10X TBS	200 mM Tris-Cl (pH: 7.6) 1.5 M NaCl
1X TBST	1X TBS 0.1% Tween-20
1X Transfer Buffer	10% 10X SDS Running Buffer 20% Methanol
10X TBE	108 g Tris Base 55 g Boric Acid 40 ml 0.5 M EDTA (pH:8.0)
%5 Blocking Solution	5% non-fat dry milk in 1X TBS-T
Laemmli Dye	250 mM Tris-Cl (pH:6.8) 8% SDS 40% Glycerol 20% β -mercaptoethanol 0.008% bromphenolblue
RIPA Lysis Buffer	750 mM NaCl 250 mM Tris (pH: 8.0) 0.1% SDS 1.0% NP-40
10X PBS	BE17-517Q, Lonza, Belgium
Non-essential Amino Acid Solution	11140-035, Gibco Invitrogen, USA
100X Penicillin-Streptomycin	15140-122, Gibco Invitrogen, USA
Dulbecco's Modified Eagle Medium (DMEM)	41965-039, Gibco Invitrogen, USA
0,05% Trypsin-EDTA	25300-054, Gibco Invitrogen, USA
Fetal Bovine Serum (FBS)	15140-122, Gibco Invitrogen, USA

Table 3.2: Antibodies.

C-Parp Ab	(human specific): CST-9541S
XBP-1 Ab:	Abcam: ab37152
ACTIN Ab:	CST-4967
RTCB Ab:	NBP1-56855
Cyclophilin	PA1-027A

Table 3.3: Drugs.

Tunicamycin:	Sigma, T7765
tert-Butyl hydroperoxide	Merck, 8.14006.0005
Oxaliplatin:	R&D 2623/50
Cisplatin:	Sigma, P4394

Table 3.4: Preparation of 10% SDS-PAGE Separating Gel.

10% separating gel	5 ml	10 ml	15 ml
ddH ₂ O	2.4	4.8	7.2
40% Acryl:Bis	1.25	2.5	3.75
1.5 M Tris pH8.8	1.25	2.5	3.75
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.002	0.004	0.006

Table 3.5: Preparation of 8% SDS-PAGE Separating Gel.

8% separating gel	5 ml	10 ml	15 ml
ddH ₂ O	2.65	5.3	7.95
40% Acryl:Bis	1	2	3
1.5 M Tris pH8.8	1.25	2.5	3.75
10% SDS	0.05	0.1	0.15
10% APS	0.05	0.1	0.15
TEMED	0.003	0.006	0.009

Table 3.6: Preparation of 5% Stacking Gel.

5% Stacking Gel	2 ml	4 ml	6 ml	8 ml
ddH ₂ O	1.46	2.92	4.38	5.84
40% Acryl:Bis	0.25	0.5	0.75	1
1 M Tris pH 6.8	0.25	0.5	0.75	1
10% SDS	0.02	0.04	0.06	0.08
10% APS	0.02	0.04	0.06	0.08
TEMED	0.002	0.004	0.006	0.008

Table 3.7: Equipments.

Centrifuges	521-1647 refrigerated, Micro Star 17R, VWR, USA 521-1646 Micro Star 17, VWR, USA 521-2844 Mini Star, VWR, USA NF400-Nuve , 02-1827
Chemiluminescence Imaging System	ChemiDoc™ XRS+ System, Bio-Rad, USA
Incubator (CO ₂)	Thermo-Scientific, USA
Electrophoresis System	1658004, Mini-PROTEAN® Bio-Rad, USA
Inverted Microscope	(Nikon, Eclipse, E100)
Laminar Flow Cabinet	Hera-Safe Class II Cabinet, Thermo Scientific, USA
Microplate Reader	Varioskan Flash, Thermo Scientific, USA
Power Supply	Bio-Rad, USA
TransBlot Transfer System	1704155, Bio-Rad, USA
Shaker	Standart Analog shaker, VWR, USA
Water Bath	Wisd, Germany

4. METHODS

HepG2, SkHep1, Snu398, Hep3B and HUH7 cell lines were used for determining RTCB expression levels. Further analysis was conducted with HepG2 (well differentiated) and Skhep1 (poorly differentiated) cell lines. Circadian clock knockouts of MDA-MD-231 cells were provided by Dr. Nuri Ozturk and Tuba Korkmaz. RTCB knockouts were provided by Dr. Nuri Ozturk. All cell lines were cultured in high glucose DMEM containing 100U/ml penicillin and 100µg/ml streptomycin antibiotics solution, 10% Fetal Bovine Serum (FBS) and 1% non essential aminoacid solution. Cells were incubated at 37°C cell culture incubator at humid environment with 5% CO₂ concentration. Cell culture medium was changed twice a week. 0.05% trypsin-EDTA solution was used to detach cells for passaging.

4.1. Sample Preparation

First, cell culture medium was removed and cell monolayer was detached by scraping with 1 ml PBS. Cell suspension was put in a 1.5 ml centrifugation tube and centrifuged at 5000 rpm for 3 minutes and supernatant was removed. Cells were lysed with ice cold RIPA buffer containing protease inhibitors, and tubes were incubated on ice for 20 minutes. Pellet was dissolved and tubes were centrifuged at 13,000 rpm for 15 minutes at 4°C. Supernatant containing protein lysate was collected and transferred into a new 1.5 ml tube.

4.2. Determining Protein Concentration

Bradford assay (Biorad Protein Assay Dye Reagent Concentrate, Cat: 500-0006) was performed for determining protein concentrations. Bovine Serum Albumin (BSA) solutions in known concentrations were used for creating a protein concentration curve to determine unknown protein concentrations of samples. Absorbance was measured at 595 nm wavelength and protein concentration differences among different samples were equalized by RIPA buffer addition. Each

sample, containing 30 µg protein was mixed with Laemmli loading buffer containing 5% β-mercaptoethanol and loaded to PAGE gel.

4.3. SDS-PAGE Gel Preparation

Cassette was prepared by bringing together the separate glasses, and 10 ml separating SDS-PAGE gel (Table 3.4 and 3.5) was poured between them. 2-propanol was added on top of the gel to prevent bubbles. After the separation gel is polymerized, stacking gel (Table 3.6) was poured and comb was inserted. Gel was placed in the electrophoresis tank after polymerization was finished. Tank was then filled with running buffer (Table 3.1), and combs were removed to create wells.

4.4. SDS-PAGE Gel Electrophoresis

SDS running buffer was used for electrophoresis. Protein samples were loaded to each well after incubation at 95°C for 10 minutes. Gel ran at 80 V until protein bands reach to separating gel. Voltage was then increased to 120 V and was stopped when loading dye reached the end.

4.5. Transferring the Protein from the Gel to the Membrane

Transfer was performed with Trans-blot System (Biorad Transblot Turbo Transfer System, Serial No: 690BR011760). 4 blotting papers and a nitrocellulose membrane was cut with the same dimensions of the SDS-PAGE gel and incubated in transfer buffer (Table 3.1). For assembling the transfer cassette; Top (-) cassette electrode (cathode) - two blotting papers - gel - blotting membrane - two blotting papers - bottom (+) cassette electrode (anode) was gathered with the same row as indicated. Trapped air was removed by using a roller. Transfer was done for 90 minutes with 12V constant voltage.

4.6. Antibody Incubation

5% blocking solution was used for blocking membranes by incubating for 1 hour at room temperature. Membranes were then incubated in primary antibody solution diluted in 5% blocking solution at 4°C overnight. Membranes are then washed with 1X TBST and incubated in horseradish peroxidase (HRP) conjugated secondary antibody solution diluted in TBST for 1 hour at room temperature.

4.7. Chemiluminescence Imaging of the Membrane

Membranes were washed with TBST three times after incubation with secondary antibody. Western Blotting Detection Kit Advansta (Cat: K-12043-D10) or and Signal Fire™ ECL Reagent (Cell Signaling Cat: 68835) chemiluminescent substrates were applied on membranes. Chemiluminescent signals were detected by Biorad Molecular Imager ChemiDoc™ XRS+with Image Lab™ Software (Serial No: 721BR04545). Each measurement was repeated for 3 times. Band intensities were evaluated with ImageJ software and bands were normalized to control protein bands. Statistics were done with Graphpad Prism 7 software. Evaluation between two groups were performed with Mann-Whitney test, whereas three and more groups were evaluated with one-way ANOVA. Statistical differences between the doses of each group at the same timepoint was evaluated by Kruskal-Wallis test. Confidence level was evaluated at 95%.

5. RESULTS

In this study, tunicamycin was applied for inducing ER stress, whereas tert-Butyl hydroperoxide (tBHP) was used for inducing oxidative stress. tBHP is a common oxidative stress inducer which acts in two ways; first, it causes alkoxyl and peroxy radicals that leads to lipid peroxidation and second, it causes glutathione peroxidase depletion [Kučera et. al. 2014]. Tunicamycin, which is a common substance for inducing ER stress is an N-linked glycosylation inhibitor, acts by blocking the first step of glycoprotein biosynthesis that leads to unfolded glycoprotein accumulation in the ER [Wang et. al. 2015; Osowski et. al. 2011]

Tunicamycin was dissolved in DMSO before application to cell culture. Oxaliplatin and cisplatin was applied for inducing DNA damage. Apoptosis was determined by evaluating cleaved poly (ADP-ribose) polymerase (c-PARP) protein levels. Western blot band intensities were calculated with ImageJ software and results were normalized to actin and cyclophilin levels.

Figure 5.1 shows protein levels of non treated hepatocellular carcinoma cell lines' (HCCs) RTCB protein levels (HCC). Further analysis was conducted with SkHep1 and HepG2 cell lines, which also show highest endogenous RTCB levels compared to other HCCs. Each cell with highest RTCB expression was chosen to reflect poorly differentiated (SkHep1) and well differentiated (HepG2) carcinomas. It is known that poorly differentiated carcinomas more invasive; thus, higher endogenous expression of RTCB in SkHep1 cell line compared to HepG2 cell line may indicate a possible correlation between RTCB and higher invasion rate.

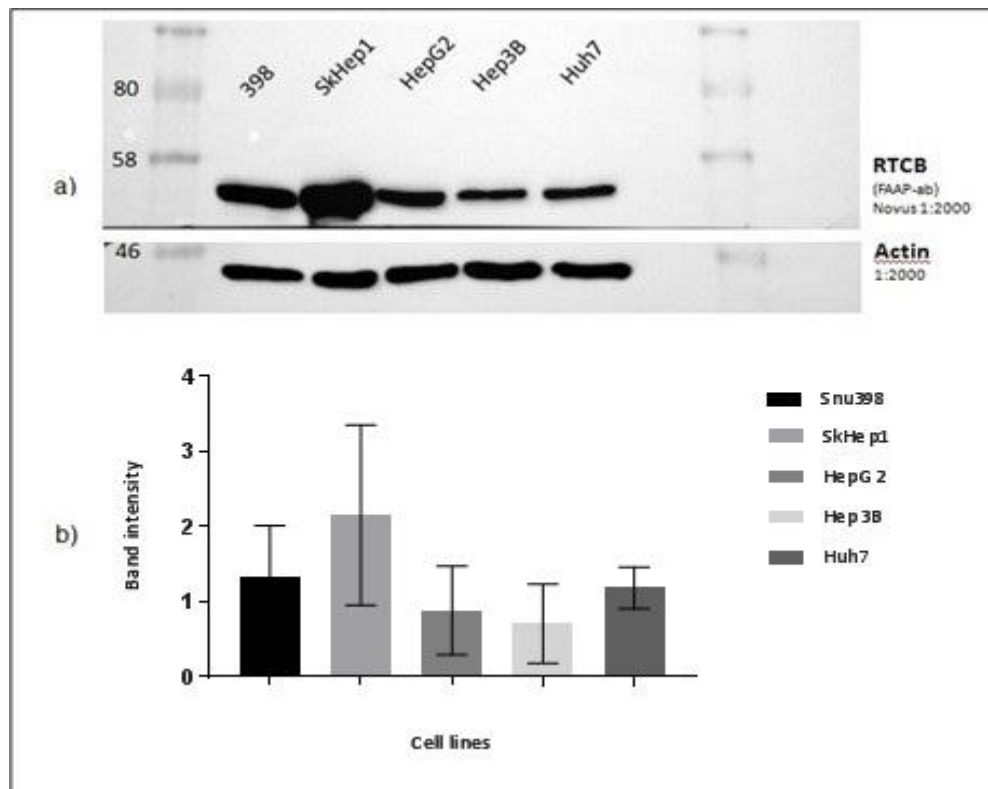


Figure 5.1: a) Immunoblot image indicating RTCB protein levels of different HCC cell lines. b) Band graphics of endogenous RTCB protein levels of HCC cell lines.

Since the aim was to find of interconnection between RTCB and DNA damage induced apoptosis, we aimed to evaluate RTCB levels after inducing apoptosis with oxaliplatin treatment. Figure 5.2 shows the apoptosis rate upon increasing dose of oxaliplatin treatment and change in RTCB protein levels. In this experiment; SkHep1 and HepG2 cells were treated with 20, 40 and 80 μ M oxaliplatin for 8 hours. It was observed that oxaliplatin treated SkHep1 cells' show significantly higher expression of both RTCB and c-PARP compared to HepG2 cells at the protein level ($P < 0,05$), however this increase in protein expression is not dose dependent. No significant difference after 20, 40 and 80 μ M oxaliplatin treatment was observed of both cell lines' RTCB and c-PARP levels ($P > 0,05$).

Altogether, there results indicate that increasing doses of oxaliplatin for 8 hours does not induce the apoptosis significantly in SkHep1 and HepG2 cells (Fig. 5.2.1a) and there is no significant change in RTCB protein levels upon oxaliplatin treatment (Fig. 5.2.1b).

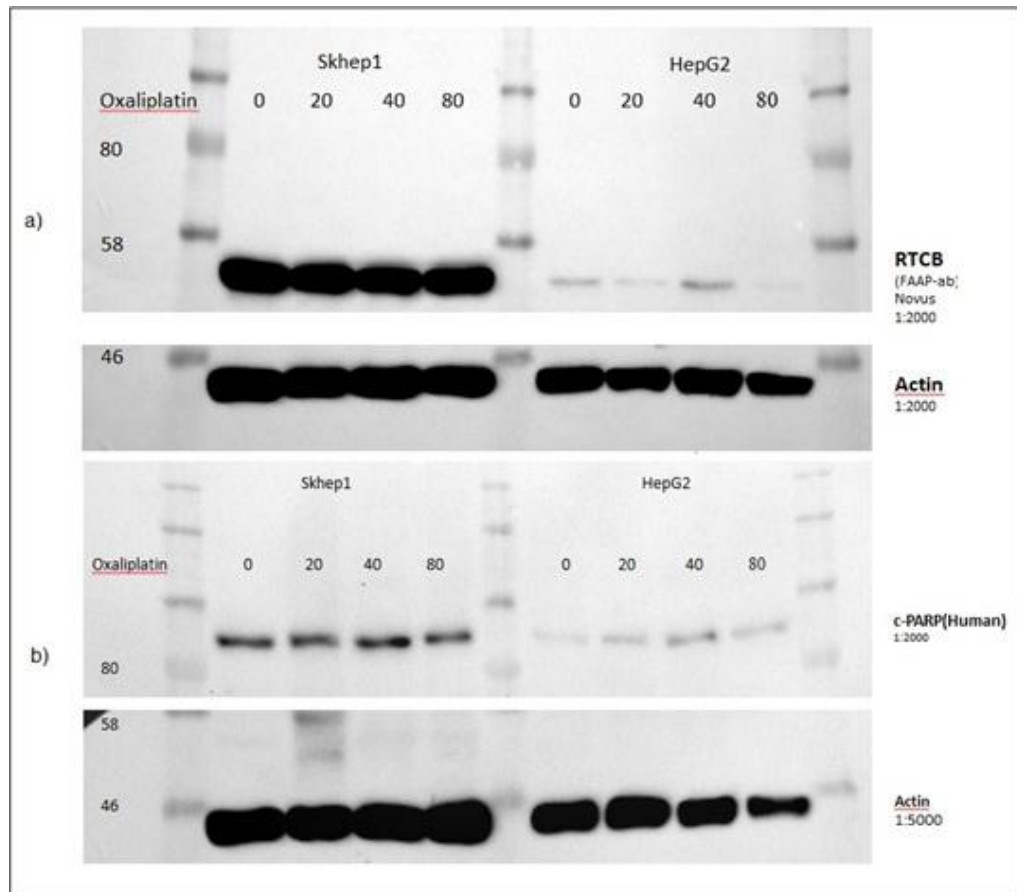


Figure 5.2: Immunoblot image of SkHep1 and HepG2 cell lines' RTCB and c-PARP proteins' expression levels after 8 hours of oxaliplatin treatment. a) Two cell lines' RTCB protein levels after oxaliplatin treatment, b) Two cell lines' c-PARP protein levels after oxaliplatin treatment.

Same experiment is then repeated with extended time of treatment; SkHep1 and HepG2 cells were treated with the same oxaliplatin for 16 hours. SkHep1 cell line's RTCB protein levels were found significantly higher than HepG2 cells' RTCB protein levels ($P < 0,05$). No difference between two cell lines' c-PARP levels was observed ($P > 0,05$) (Figure 5.3). However, as seen on Figure 5.3; compared to SkHep1 cell line, c-PARP levels of HepG2 upon 80 μM oxaliplatin treatment is higher, which may be related to the different p53 status of these cell lines; HepG2 is a p53 wild-type HCC cell line whereas SkHep1 is a p53 mutant cell line which has a base substitution mutation [Yoshikawa, Nagashima et. al. 1999].

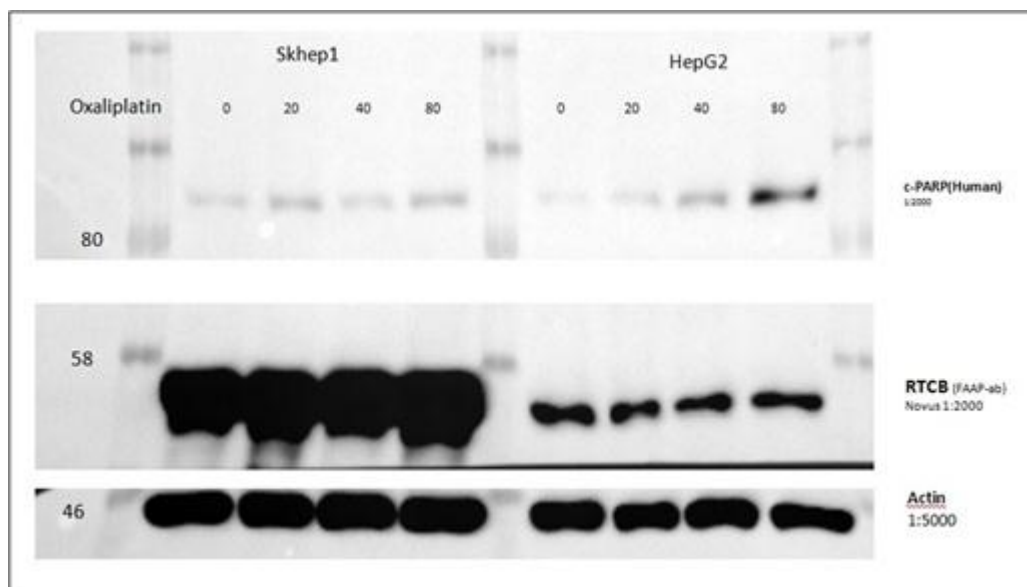


Figure 5.3: Immunoblot image of oxaliplatin treatment for 16 hours. No significant change in RTCB levels among each cell type did not observed. c-PARP levels did not alter among each group.

In order to figure out if RTCB protein levels will be effected by cellular stress induced apoptosis, ER stress and oxidative stress were induced in SkHep1 and HepG2 cells in two different concentrations. 1 and 2 μ M tunicamycin were used for ER stress induction; 20 and 40 μ M tBHP were used to induce oxidative stress for 8 hours. No difference between both cell lines' c-PARP protein levels was found after tunicamycin treatment ($P > 0,05$). Difference between c-PARP levels of SkHep1 cells after 1 and 2 μ M tunicamycin was found significant compared to non treated cells ($P < 0,05$), however HepG2 cells' c-PARP levels did not show a significant difference upon treatment ($P > 0,05$). tBHP did not cause any statistical difference at c-PARP protein levels of both cell lines ($P > 0,05$). c-PARP protein levels were found significantly different between 0, 20 and 40 μ g/ml tBHP treatment in HepG2 cells ($P < 0,05$)

Results demonstrated that tunicamycin induces apoptosis in both SkHep1 and HepG2 with dose dependent manner; however, tBHP induced apoptosis in only SkHep1 cells but not in HepG2 cells. RTCB levels did not change significantly upon treatment of tunicamycin or tBHP. Change in RTCB protein levels were found both non significant for tunicamycin and tBHP treatment between SkHep1 and HepG2

cells, and RTCB protein expression levels according to different dose applications did not show statistical significance ($P > 0,05$).

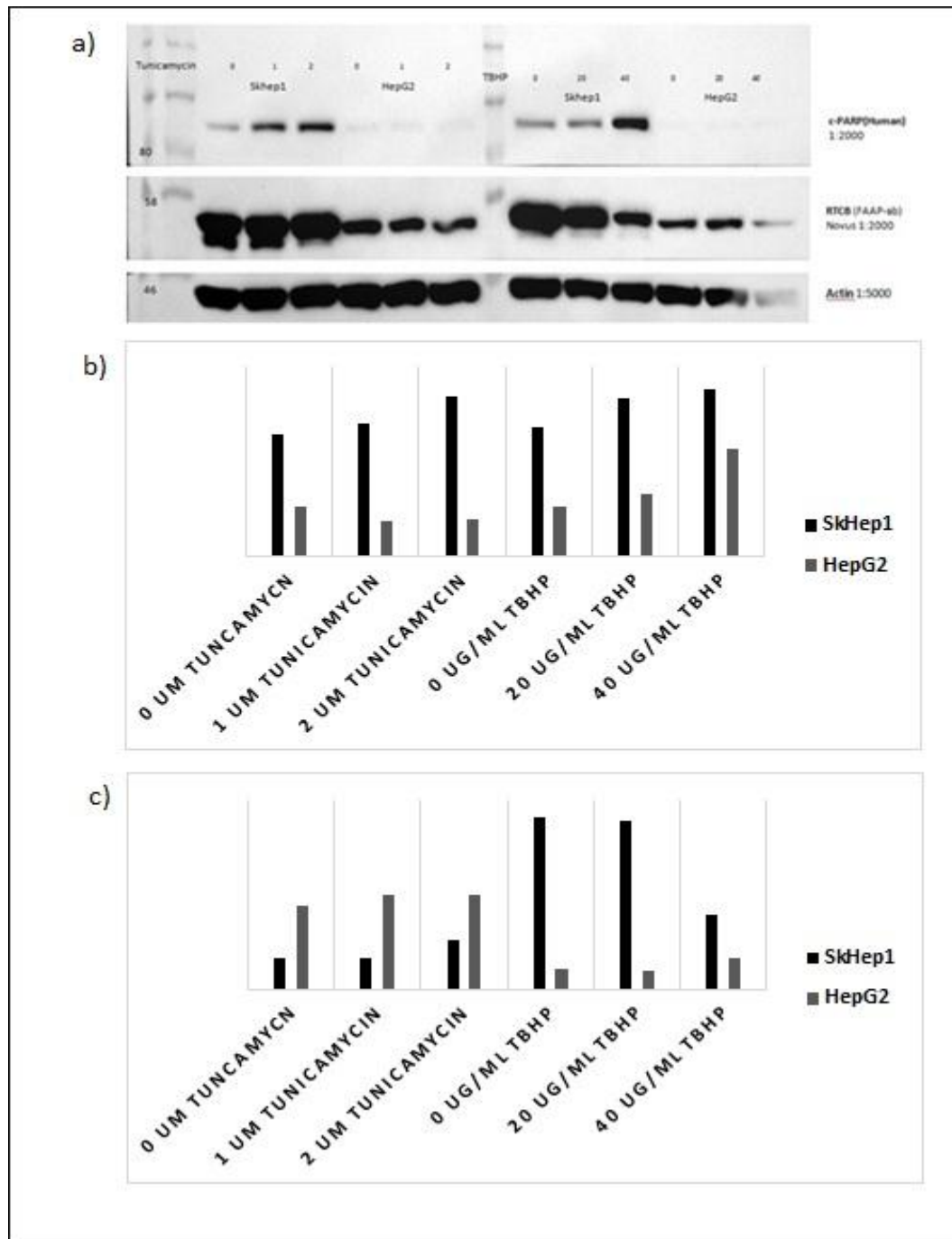


Figure 5.4: a) Immunoblot image indicating effects of tunicamycin and tBHP for 8 hours on c-PARP and RTCB protein levels. Apoptosis was evaluated with c-PARP antibody at the protein level. b) Graphical representation of SkHep1 and HepG2 cell lines' c-PARP levels after tunicamycin and tBHP treatment. c) Graphical representation of SkHep1 and HepG2 cell lines' RTCB levels after tunicamycin and tBHP treatment.

Then, SkHep1 cells were treated with 20,30 and 40 μ M tBHP and 2 μ M tunicamycin separately, for 8 hours for determining optimum dose of tBHP. It was found that effect of tBHP on c-PARP expression at the protein level is significant ($p < 0,05$), and 2 μ M tunicamycin treated cells' c-PARP protein levels are found statistically significant compared to non treated cells and DMSO treated cells as control ($P < 0,05$). However, as indicated in Figure 5.5, we could not detect any significant difference in RTCB protein levels between groups ($P > 0,05$).

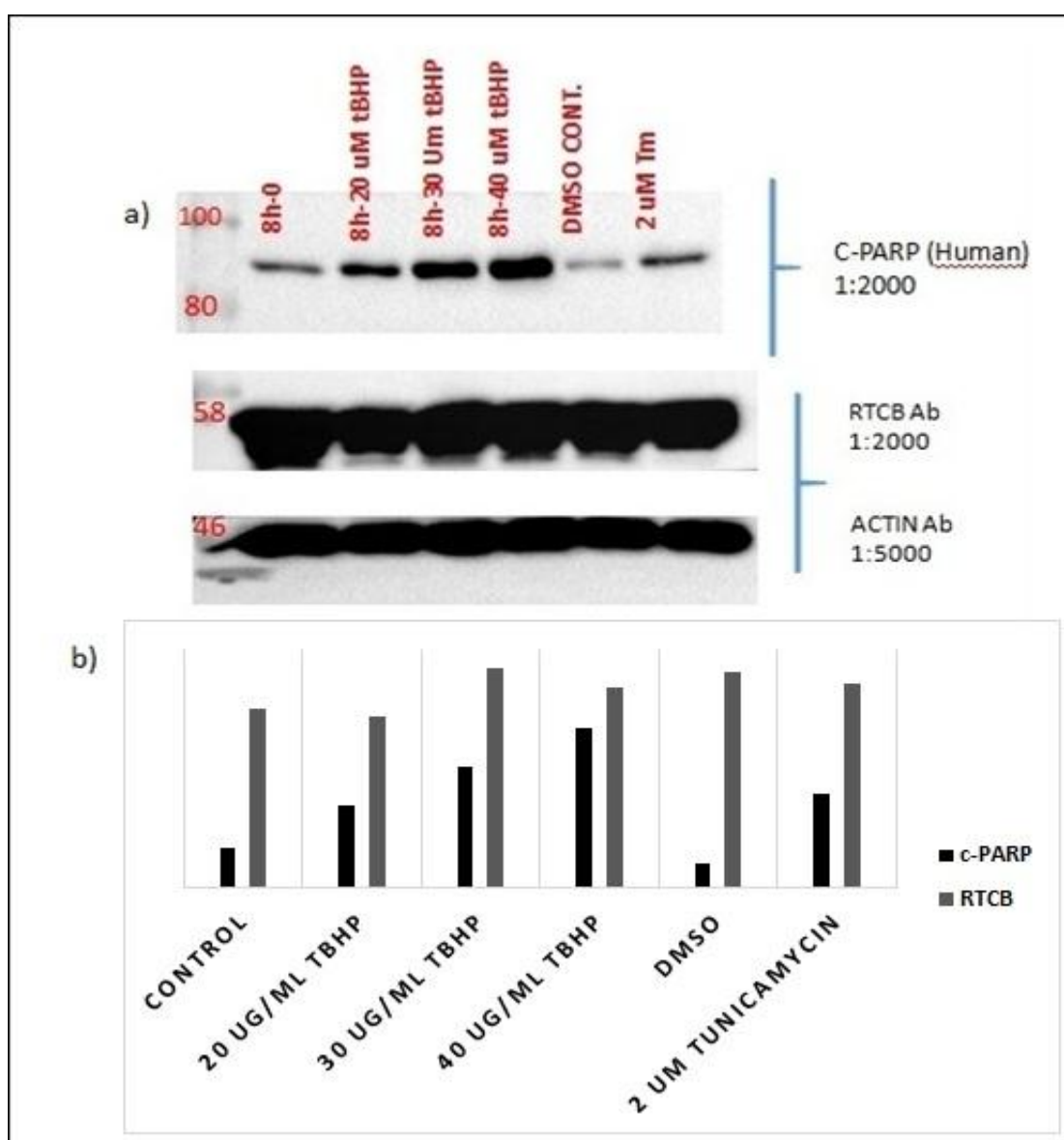


Figure 5.5: a) Immunoblot image of tBHP at 20, 30 and 40 μ M concentrations and tunicamycin at 2 μ M concentration for 8 hours on SkHep 1 cell line's RTCB and c-PARP levels. b) Graphical representation of RTCB and protein levels of SkHep1 cells' after tBHP at various concentrations and 2 μ M tunicamycin treatment for 8 hours.

It is both well known that CCGs play role on cells' ability to cope with stress and effect of RTCB has effect on UPR by splicing Xbp1. In order to see if RTCB protein levels are influenced by CCGs' status or if it's expression is under control of p53, p53 knockout immortalized mouse skin fibroblasts and triple knockout (Cry1, Cry2 and p53 knockout) immortalized mouse skin fibroblast cells were treated with 0,5 and 2 μ M tunicamycin (Figure 5.6 and Figure 5.7) and 20, 30, 40 μ M tBHP (Figure 5.7). Changes of RTCB protein levels' on both timepoints between groups remained non significant ($P > 0,05$).

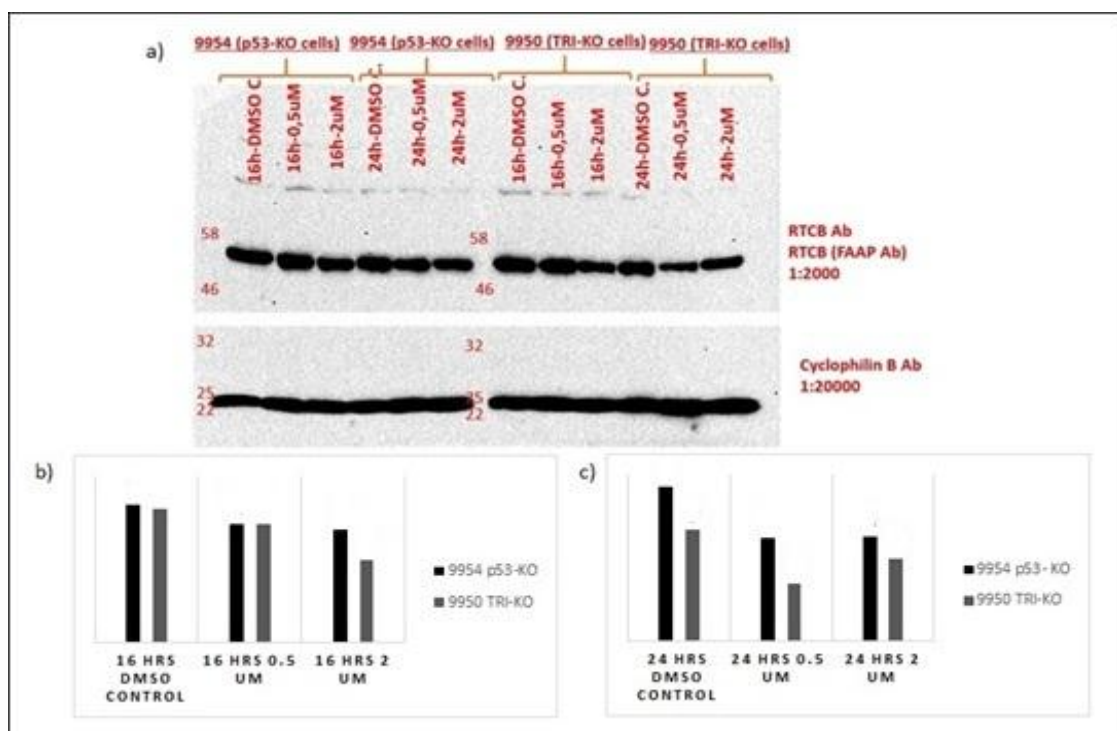


Figure 5.6: a) Immunoblot image of 0,5 and 2 μ M tunicamycin treatment's effect on p53 knockout and triple knockout mouse skin fibroblast cell line's RTCB levels. DMSO was used as control. Cyclophilin was used as internal control. b) RTCB protein levels of tunicamycin treated P53-KO and TRI-KO cells for 16 hours. c) RTCB protein levels of tunicamycin treated P53-KO and TRI-KO cells for 24 hours.

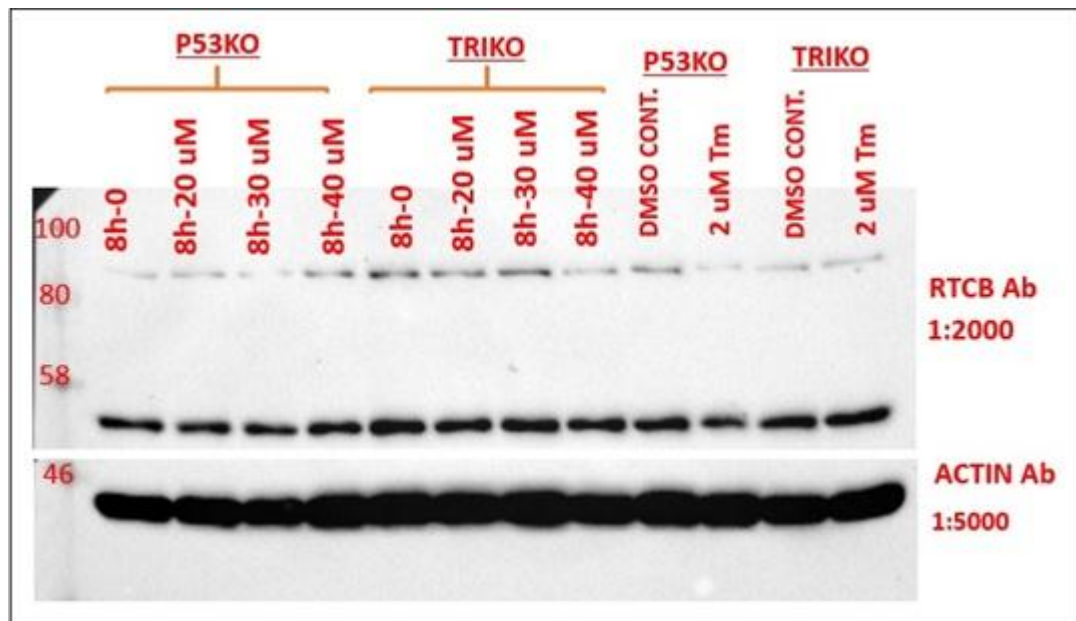


Figure 5.7: Immunoblot image indicating different concentrations of tBHP and 2 μ M tunicamycin on p53 knockout and triple knockout mouse skin fibroblast cell line's RTCB and c-PARP levels after 8 hours treatment. DMSO was used as control. Actin was used as internal control.

Also RTCB levels of wild-type, triple knockout, Clock knockout and Bmal1 knockout MDA-MB-231 cells were checked in addition to p53 knockout and triple knockout mouse skin fibroblast cells (Figure 5.8) in order to see the link between RTCB and CCG proteins. No significant difference between p53 knockout and triple knockout cells' RTCB levels upon treatment with tBHP at different concentrations for 8 hours was detected ($P > 0,05$). Difference between p53 knockout and triple knockout cells' RTCB protein levels after tunicamycin treatment was also non significant ($P > 0,05$).

No significant difference between p53 knockout and triple knockout mouse skin fibroblasts' RTCB levels were detected ($P > 0,05$). Difference between RTCB protein levels of MDAMB231 cells were found significant ($P = 0,0001$). Taken together, these results indicate that RTCB expression is similar in wild-type and circadian clock knockouts, thus we can conclude that for both cell lines', RTCB protein levels are not directly influenced by CCG proteins or p53 status.

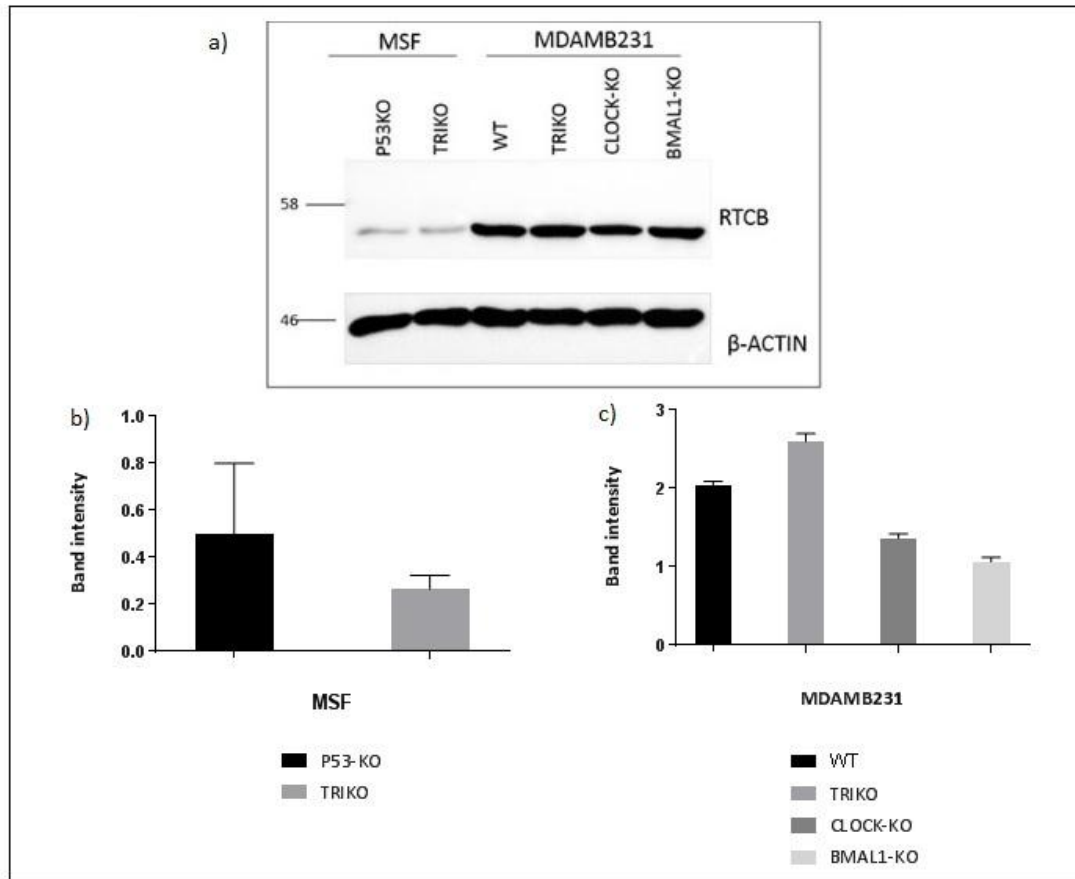


Figure 5.8: a) Immunoblot image of RTCB protein level in wild type and Clock knockout cells. Western blot was performed by Handan Emiřođlu. b) Bar graphic of RTCB protein levels of p53 knockout and triple knockout mouse skin fibroblasts. c) Bar graphic of RTCB protein levels of wild type, triple knockout, clock genes knockout and BMAL1 knockout MDAMB231 cells.

Since Xbp1 splicing depends on RTCB activation in UPR, we checked RTCB protein levels and spliced Xbp1 levels of RTCB wild type HEK293T and RTCB knockout HEK293T cells after inducing ER stress with tunicamycin (Figure 5.9). RTCB levels compared with wild type Hek-293T cells show that knockout process is accomplished (Figure 5.9a), RTCB levels of knockout cells were found significantly lower than wild type cells ($P < 0,0001$). Decrease in Xbp1 levels were found not significantly decreased in RTCB knockout cells after ER stress induction (Figure 5.9b) ($P > 0,05$), however this may be due do the lack of the repetition of analysis.

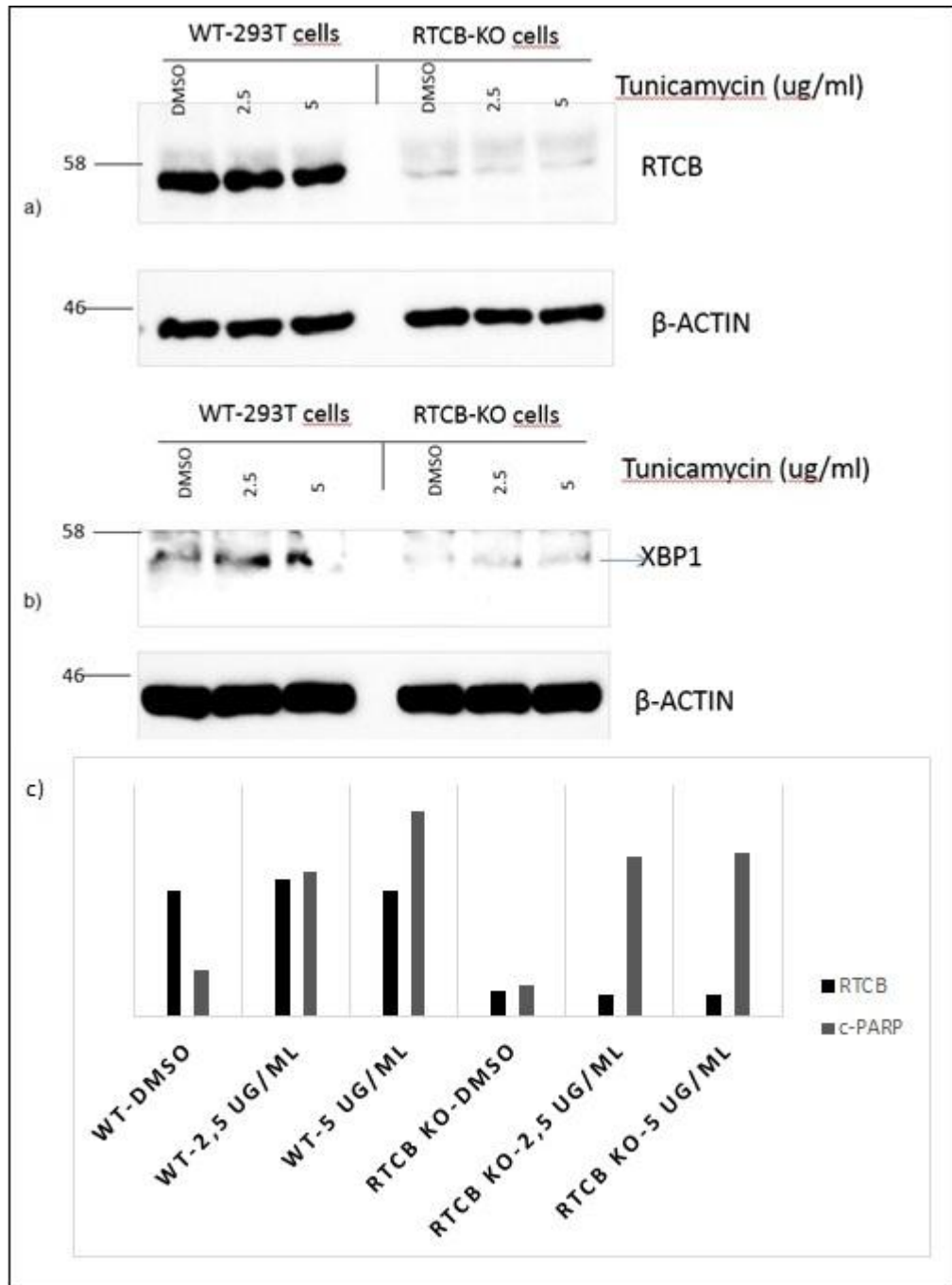


Figure 5.9: Immunublot images of a) RTCB b) Xbp1 protein levels of wild type and RTCB knock-out cells after tunicamycin treatment. Western blot was performed by Handan Emiřođlu. c) Bar graphics of RTCB and c-PARP protein expressions of cells after treatment at doses indicated.

As the next step, wild type and RTCB knockout HEK293T cells' response to oxidative and ER stress was evaluated by inducing cellular stress with various doses of tBHP and tunicamycin. 6 hours of tunicamycin and tBHP treatment indicate RTCB does not involve in ER stress or oxidative stress induced apoptosis even though it

plays role in UPR in ER stress. Apoptosis rate is measured with c-PARP at protein level and difference after tBHP and tunicamycin treatments at 2 different doses resulted in c-PARP levels of both between groups and among each group is found non significant ($P > 0,05$). This data shows that cellular stress induced apoptosis is not effected by RTCB status.

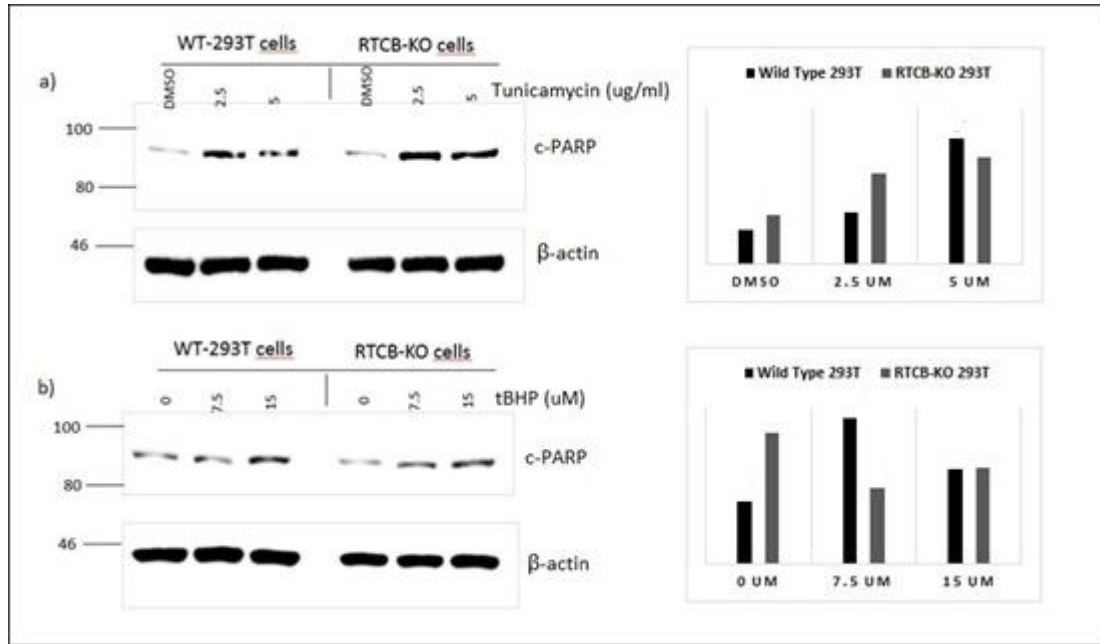


Figure 5.10: Immunoblot images of c-PARP in a) 2,5 and 5 ug/ml tunicamycin b) 7,5 and 15 uM tBHP treated WT and RTCB-KO HEK-293T cells. Western blot performed by Handan Emiřođlu. c) Bar graphics of tunicamycin and tBHP treated wild type and RTCB knockout cells' c-PARP levels.

As if it was how we hypotesized that RTCB plays role in DNA damage induced apoptosis; its' absence should have effect on cells' apoptotic process, thus c-PARP protein levels. RTCB wild type and knockout HEK293T cells were treated with cisplatin for 24 hours in order to induce apoptosis (Figure 5.11). Difference between c-PARP protein levels of wild type and RTCB knockout HEK293T cells were found non significant ($p > 0,05$). Difference of RTCB protein levels between 5 and 10 μ M cisplatin treatment also were not found significant ($p > 0,05$).

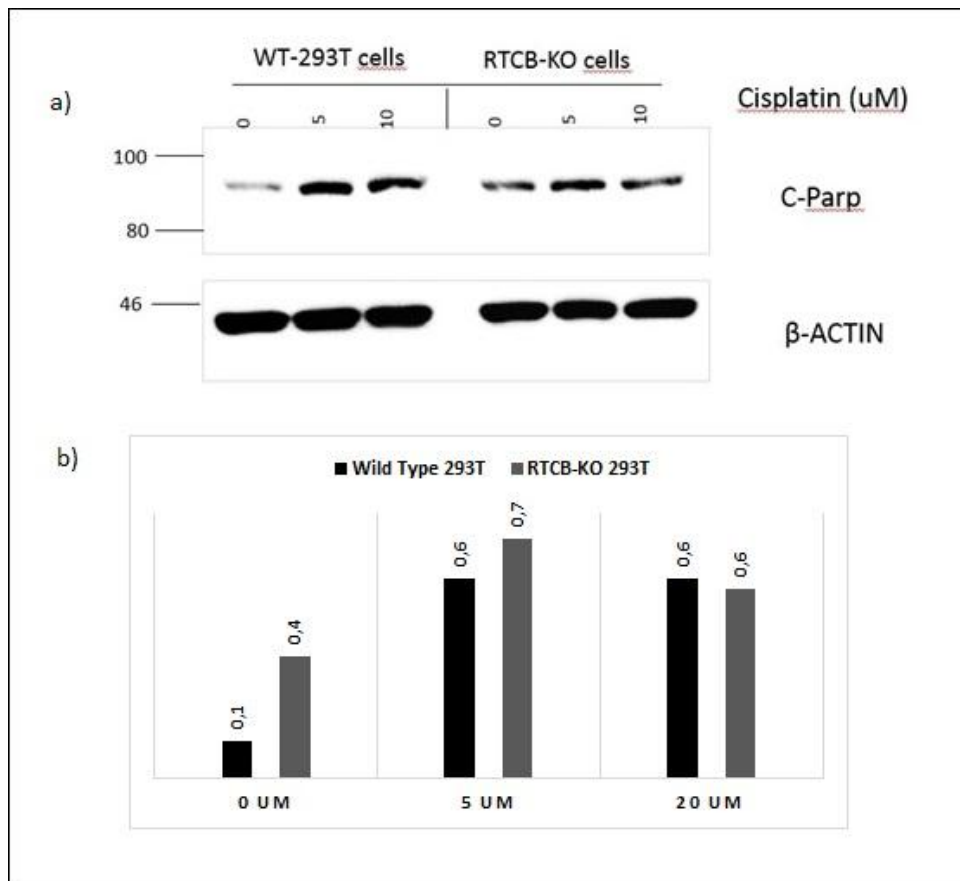


Figure 5.11: Immunoblot images of a) c-PARP levels after 5 and 10 μ M cisplatin treatment of wild type and RTCB knock-out cells.
b) Bar graphics of c-PARP levels after cisplatin treatment of cells.

6.DISCUSSION

Previous studies published in 2011 revealed that RTCB ligase directly seals 2',3'-cyclic phosphate and 5'-OH ends, and tRNA ligase Trl1 deficient *Escherichia coli*, RtcB is sufficient for tRNA splicing in vivo [Tanaka and Shuman, 2011; Tanaka et. al. 2011]. Furthermore, RtcB can also catalyse HAC1 mRNA splicing in UPR in case of Trl1 deficiency [Tanaka et. al. 2011]. Role of RTCB on UPR in *C. Elegans* has also studied; it was revealed that RTCB-1 is necessary for xbp-1, which is HAC1 homolog's mRNA splicing, and ligase activity of *C. Elegans* RTCB-1 is required for neuroprotection [Ray et al. 2014]. A recent study shows that ribosomal 16S rRNA is structurally modified in response to stress conditions by removing 3' terminus of the 16S rRNA in *E.coli*. By this process, de novo ribosome subpopulations translate the mRNAs that are only processed likewise. When the organism overcomes the stress, modified ribosomes undergo re-ligation process in which the 3' terminus is added and become typical ribosomes. This re-ligation process is occurred by the activity of RtcB in *E.coli* [Temmel et. al. 2017]. Since it is known that selective translational inhibition is one of the responses against ER stress, RTCB may also function in re-ligation of ribosomes in eukaryotes together with its Xbp1 splicing function in ER stress. Another study revealed that *Escherichia coli* RtcB also can splice the 3-phosphate and 5'hydroxyl DNA break [Das et. al. 2013]. RNA ligation and repair function of RTCB lets us to consider the repair potential of RTCB of 3'PO₄ – 5'OH DNA breaks. It is known that RTCB can catalyze the 3'PO₄ – 5'OH ends of DNA break which is also called dirty end; however this is the only study indicating the DNA repair role of RTCB [Das et. al. 2014]. Possible DNA repair functions of RTCB should be investigated with further studies. According to a study, Dnase II is the enzyme which leads 3'PO₄ – 5'OH DNA break which is hypothetically ligated and repaired by RTCB. If RTCB can repair dirty end DNA break [Didenko, 2011], induction of this type of break may activate RTCB expression in order to repair DNA.

Eventhough well known functions of RTCB include tRNA and mRNA splicing and unconventional splicing of Xbp1 mRNA; RTCB ligase has other functions in

cellular processes such as inhibiting axon growth [Tanaka et. al. 2011; Das et. al. 2013; Huang and Pommier, 2013]. Until date, the role of RTCB in stress induced DNA break repair in mammals is not known.

Here, the aim of our study to reveal possible functions of mammalian RTCB on DNA damage, ER stress and oxidative stress in vitro. In this study, we evaluated RTCB on the protein level and revealed that RTCB levels did not alter upon cellular stress induced apoptosis. However, we did not perform gene expression analysis in this study; thus, there is a possibility of gene expression level alteration of RTCB. Furthermore, studies performed with p53 knockout and triple knockout (Cry1, Cry2 and p53) indicated that RTCB protein level does not show neither p53 nor clock genes dependent gene expression pattern. Thus, it can be concluded that RTCB protein level is not p53 or CCG dependent. Also, because of the activity of RTCB depends on archease function; archease protein level should be evaluated in addition to RTCB-archease complex formation to reveal this complex is clearly do not play a role in stress induced apoptosis.

It is known that well differentiated cancer cells are less aggressive than poorly differentiated cancer cells. SkHep1 represents the poorly differentiated cells and HepG2 represents the well differentiated cells in this study. Differences between endogenous expression of RTCB between Skhep1 and HepG2 might indicate a link between RTCB and invasion rate in cancer cells. In our study, endogenous expression of RTCB is found higher in SkHep1 cells in compared with HepG2 cells; however further studies are needed in order to find out if RTCB might influence the growth and survival. Although HepG2 cells are p53 wild type, SkHep1 cells are p53 null and p53 has pivotal role in initiating apoptosis; in this study, SkHep1 showed more apoptosis rate than HepG2 after ER stress and oxidative stress inducer treatments; thus it may be concluded that there may be an interconnection between RTCB level and sensitivity to stress conditions. It is already known that ER stress is directly linked with RTCB levels since it plays role in Xbp-1 splicing. ER stress and oxidative stress are connected with each other; oxidative stress can trigger ER stress by generating ROS and altering redox state [Cao and Kaufman, 2014]. Levels of tBHP doses which we used in this study may be higher than the treshold that

mitochondria might directly activate intrinsic apoptosis levels rather than interacting with ER and resulting in ER stress associated RTCB protein expression.

Also oxaliplatin treatment experiments of Skhep1 and HepG2 in both 8 and 16 hours show that, despite of using oxaliplatin with increasing doses, both cell lines did not go under apoptosis except 80 μ M oxaliplatin treatment in HepG2 for 16 hours. Absence of p53 in Skhep1 may explain the low apoptosis level in high doses and duration of treatment. A study about HepG2 and oxaliplatin resistance reveals that oxaliplatin treatment induces autophagy which increases the resistance of HepG2 against oxaliplatin [Du et. al. 2012]. Thus, to induce apoptosis significantly, different DNA damaging agents may be used. Another solution may be treating cells for longer durations, using higher doses of oxaliplatin, or using an autophagy inhibitor together with oxaliplatin.

REFERENCES

- Breckenridge D. G., Germain M., Mathai J. P., Nguyen M., Shore G. C., (2013), "Regulation of apoptosis by endoplasmic reticulum pathways", *Oncogene*, 24;22(53),8608-18.
- Brozovic A., Fritz G., Christmann M., Zisowsky J., Jaehde U., Osmak M., Kaina B., (2004), "Long-term activation of SAPK/JNK, p38 kinase and fas-L expression by cisplatin is attenuated in human carcinoma cells that acquired drug resistance", *International Journal of Cancer*, 20;112(6),974-85.
- Cao S. S., Kaufman R. J., (2014), "Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease", *Antioxidants and Redox Signaling*, 20;21(3),396-413.
- Chaitanya G. V., Alexander J. S., Babu P. P., "PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. *Cell Communication and Signaling*, 8, 31.
- Christmann M., Tomicic M. T., Roos W. P., Kaina B., (2003), "Mechanisms of human DNA repair: an update", *Toxicology*, 15;193(1-2),3-34.
- Das U., Chakravarty A. K., Remus B. S., Shuman S. (2013), "Rewriting the rules for end joining via enzymatic splicing of DNA 3'-PO₄ and 5'-OH ends", *Proceedings of the National Academy of Sciences of the United States of America*, 110(51),20437-20442.
- Das U., Chauleau M., Ordonez H., Shuman S., (2014), "Impact of DNA3'pp5'G capping on repair reactions at DNA 3' ends", *Proceedings of the National Academy of Sciences of the United States of America*, 5;111(31),11317-22.
- Desai K. K., Beltrame A. L., Raines R. T., (2015), "Coevolution of RtcB and Archease created a multiple-turnover RNA ligase", *RNA*, 21(11),1866 - 1872.
- Desai K. K., Bingman C. A., Phillips G. N., Raines R. T., (2013), "Structures of the Noncanonical RNA Ligase RtcB Reveal the Mechanism of Histidine Guanylylation", *Biochemistry*, 52(15),2518-2525.
- Desai K. K., Cheng C. L., Bingman C.A., Phillips G. N., Raines R. T., (2014), "A tRNA splicing operon: Archease endows RtcB with dual GTP/ATP cofactor specificity and accelerates RNA ligation", *Nucleic Acids Research*, 42(6),3931-3942.
- Didenko V., (2011), "5'OH DNA breaks in apoptosis and their labeling by topoisomerase-based approach", *Methods in Molecular Biology*, 682,77-87.

Du H., Yang W., Chen L., Shi M., Seewoo V., Wang J et. al., (2012), "Role of autophagy in resistance to oxaliplatin in hepatocellular carcinoma cells" *Oncology Reports*, 27(1),143-50.

Elmore S., (2007), "Apoptosis: A Review of Programmed Cell Death", *Toxicologic pathology*, 35(4),495-516.

Farooqi A. A., Li K-T., Fayyaz S., et al., (2015), "Anticancer drugs for the modulation of endoplasmic reticulum stress and oxidative stress", *Tumour Biology*, 36(8),5743-5752.

Filipowicz W., (2014), "Making ends meet: a role of RNA ligase RTCB in unfolded protein response", *The EMBO Journal*, 33(24),2887-2889.

Fuchs Y., Steller H., (2015), "Live to die another way: modes of programmed cell death and the signals emanating from dying cells", *Nature reviews Molecular cell biology*, 16(6),329-344.

Huang S. N., Pommier Y., (2013), "Cross-over of RNA 3'-phosphate ligase into the DNA World", *Proceedings of the National Academy of Sciences of the United States of America*, 110(51),20354-20355.

Hughes M., Deharo L., Pulivarthy S. R., Gu J., Hayes K., Panda S., Hogenesch J. B. (2007), "High-resolution time course analysis of gene expression from pituitary" *Cold Spring Harbor Symposia on Quantitative Biology*, 2007;72,381–6.

Jamieson E. R., Lippard S. J., (1999), "Structure, Recognition, and Processing of Cisplatin-DNA Adducts", *Chemical Reviews*, 8;99(9),2467-98.

Jurkin J., Henkel T., Nielsen A. F., et al., (2014), "The mammalian tRNA ligase complex mediates splicing of XBP1 mRNA and controls antibody secretion in plasma cells", *The EMBO Journal*, 33(24),2922-2936.

Kerr J., F.R., Wyllie A. H., Currie A. R., (1972), "Apoptosis: A Basic Biological Phenomenon with Wide-ranging Implications in Tissue Kinetics", *British Journal of Cancer*, 26(4),239-257.

Kitamura M., (2008), "Endoplasmic reticulum stress and unfolded protein response in renal pathophysiology: Janus faces", *American Journal of Physiology – Renal Physiology*, 295 (2) F323-F334.

Kosmaczewski S. G., Edwards T. J., Han S. M., et al., (2014), "The RtcB RNA ligase is an essential component of the metazoan unfolded protein response", *EMBO Reports*, 15(12),1278-1285.

Kosmaczewski S. G., Han S. M., Han B., et al., (2015), "RNA ligation in neurons by RtcB inhibits axon regeneration", *Proceedings of the National Academy of Sciences of the United States of America*, 112(27),8451-8456.

Kučera O., Endlicher R., Roušar T., et al., (2014), "The Effect of tert-Butyl Hydroperoxide-Induced Oxidative Stress on Lean and Steatotic Rat Hepatocytes In Vitro", *Oxidative Medicine and Cellular Longevity*, 2014,752506.

Lips J., Kaina B., (2001), "DNA double-strand breaks trigger apoptosis in p53-deficient fibroblasts", *Carcinogenesis*, 22(4),579-85.

Liu D., Ke Z., Luo J., (2016); "Thiamine Deficiency and Neurodegeneration: the Interplay Among Oxidative Stress, Endoplasmic Reticulum Stress, and Autophagy", *Molecular Neurobiology* [Epub ahead of print].

Lockshin R. A., Williams C. M., (1964), "Programmed cell death — II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkworms", *Journal of Insects Physiology*, 10, 643–649.

Lu Y., Liang F-X., Wang X., (2014), "A synthetic biology approach identifies the mammalian UPR RNA ligase RtcB", *Molecular Cell*, 55(5),758-770.

McIlwain D. R., Berger T., Mak T. W., (2013), "Caspase Functions in Cell Death and Disease", *Cold Spring Harbor Perspectives in Biology*, 5(4),a008656.

Nagasaka A., Kawane K., Yoshida H., Nagata S., (2010), "Apaf-1-independent programmed cell death in mouse development", *Cell Death and Differentiation*, 17, 931–941.

Norbury C. J., Zhivotovsky B., (2004), "DNA damage-induced apoptosis", *Oncogene*, 23(16),2797-808.

Osowski C., Urano F., (2011), "Measuring ER stress and the unfolded protein response using mammalian tissue culture system", *Methods in enzymology*. 490,71-92.

Popow J., Jurkin J., Schleiffer A., Martinez J., (2014), "Analysis of orthologous groups reveals Archease and DDX1 as tRNA splicing factors", *Nature*. 511(7507),104-107.

Ray A., Zhang S., Rentas C., Caldwell K. A., Caldwell G. A., (2014), "RTCB-1 Mediates Neuroprotection via XBP-1 mRNA Splicing in the Unfolded Protein Response Pathway", *The Journal of Neuroscience*, 34(48),16076-16085.

Redza-Dutordoir M., Averill-Bates D. A., (2016), "Activation of apoptosis signalling pathways by reactive oxygen species", *Biochimica et Biophysica Acta*, (12),2977-2992.

Rodriguez, J., Lazebnik, Y., (1999), "Caspase-9 and APAF-1 form an active holoenzyme", *Genes & Development*, 13, 3179–3184.

Roos W. P., Thomas A. D., Kaina B., (2016), "DNA damage and the balance between survival and death in cancer biology", *Nature Reviews Cancer*, 16(1),20-33.

Ryoo H. D., (2016), "Long and short (timeframe) of endoplasmic reticulum stress-induced cell death", *FEBS Journal*, 283(20),3718-3722.

Sancar A., Lindsey-Boltz L. A., Gaddameedhi S., et al., (2015), "Circadian Clock, Cancer, and Chemotherapy" *Biochemistry*, 54(2),110-123.

Sancar A., Lindsey-Boltz L. A., Kang T-H, Reardon J. T., Lee J. H., Ozturk N., (2010), "Circadian Clock Control of the Cellular Response to DNA Damage", *FEBS letters*, 584(12),2618-2625.

Schröder M., Kaufman R. J., (2005), "ER stress and the unfolded protein response", *Mutation Research*, 569(1-2),29-63.

Schuler M., Green D. R., (2001), "Mechanisms of p53-dependent apoptosis", *Biochemical Society Transactions*, 29(Pt 6),684-8.

Srivastava M., Scherr H., Lackey M., Xu D., Chen Z., Lu, J., & Bergmann A. (2007). ARK, the Apaf-1 related killer in *Drosophila*, requires diverse domains for its apoptotic activity. *Cell Death and Differentiation*, 14(1), 92–102.

Tanaka N., Chakravarty A. K., Maughan B., Shuman S., (2011), "Novel Mechanism of RNA Repair by RtcB via Sequential 2',3'-Cyclic Phosphodiesterase and 3'-Phosphate/5'-Hydroxyl Ligation Reactions", *The Journal of Biological Chemistry*. 286(50),43134-43143.

Tanaka N., Meineke B., Shuman S. (2011), "RtcB, a Novel RNA Ligase, Can Catalyze tRNA Splicing and HAC1 mRNA Splicing in Vivo" *The Journal of Biological Chemistry*. 286(35),30253-30257.

Tanaka N., Shuman S., (2011), "RtcB Is the RNA Ligase Component of an *Escherichia coli* RNA Repair Operon", *The Journal of Biological Chemistry*, 286(10),7727-7731.

Tang T. H., Chang C. T., Wang H. J., Erickson, J. D., Reichard, R. A., Martin, A. G., et al., (2013); "Oxidative stress disruption of receptor-mediated calcium signaling mechanisms", *Journal of Biomedical Science*, 20(1),48.

Tata, J. R., (1966), "Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture", *Developmental Biology*, 13, 77–94.

- Temmel, H., Müller, C., Sauert, M., Vesper, O., Reiss, A., Popow, J., et. al. (2017), "The RNA ligase RtcB reverses MazF-induced ribosome heterogeneity in *Escherichia coli*", *Nucleic Acids Research*, 45(8), 4708–4721.
- Tenzer A., Zingg D., Riesterer O., Vuong V., Bodis S., Pruschy M., (2002), "Signal transduction inhibitors as radiosensitizers", *Current Medicinal Chemistry: Anticancer Agents*, 2(6),727-42.
- Thornberry N. A., Lazebnik Y., (1998), "Caspases: enemies within", *Science*, 28;281(5381),1312-6.
- Wang, H., Wang, X., Ke, Z.-J., Comer, A. L., Xu, M., Frank, J. et. al., (2015), "Tunicamycin-induced Unfolded Protein Response in the Developing Mouse Brain", *Toxicology and Applied Pharmacology*, 283(3), 157–167.
- Wang J., Yang X., Zhang J., (2016), "Bridges between mitochondrial oxidative stress, ER stress and mTOR signaling in pancreatic β cells", *Cell Signaling*, 28(8),1099-104.
- Woynarowski J. M., Faivre S., Herzig M. C., Arnett B., Chapman W. G., Trevino A. V., Raymond E., Chaney S. G., Vaisman A., Varchenko M., Juniewicz P. E., (2000), "Oxaliplatin-induced damage of cellular DNA", *Molecular Pharmacology*, 58(5),920-7.
- Xiang C., Wang Y., Zhang H., Han F., (2017), "The role of endoplasmic reticulum stress in neurodegenerative disease", *Apoptosis*, 22(1),1-26.
- Xu C., Bailly-Maitre B., Reed J. C., (2005), "Endoplasmic reticulum stress: cell life and death decisions", *Journal of Clinical Investigation*, 115(10),2656-2664.
- Xu D., Li Y., Arcaro M., Lackey M., Bergmann A., (2005), "The CARD-carrying caspase Dronc is essential for most, but not all, developmental cell death in *Drosophila*", *Development* 132(9),2125–34.
- Yoshikawa H., Nagashima M., Khan M. A., McMenamin M. G., Hagiwara K., Harris C. C., (1999), " Mutational analysis of p73 and p53 in human cancer cell lines", *Oncogene*, 3;18(22),3415-21.

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APPENDICES

Appendix A: Copyright Permission

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